Molecular genetics
of
nonspecific X-linked mental retardation

ISBN: 90-9015121-4
Druk: Printpartners Ipskamp, Enschede
Omslag: Ad van den Broek, Wilson Design, Uden

The research described in this thesis was carried out at the Department of Human Genetics, University Medical Centre Nijmegen, The Netherlands. Financial support was received from the Deutsche Forschungsgemeinschaft, ZorgOnderzoek Nederland, the Ter Meulen Fund (Royal Netherlands Academy of Arts and Sciences), and Hersenstichting Nederland.

Molecular genetics of nonspecific X-linked mental retardation

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
woensdag 7 november 2001
des namiddags om 1.30 uur precies

door

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geboren op 10 mei 1971
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voor mijn ouders

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ABBREVIATIONS

AAMD	American Association of Mental Deficiency
ACh	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ARHGEF6	Rho GEF protein 6
ATR-X	X-linked mental retardation syndrome with α-thalassemia
BDNF	brain-derived neurotrophic factor
bp	base-pairs
Cdc42	cell division cycle 42
CBP	CREB-binding protein
CH	calponin homology
CHM	choroideremia
CLS	Coffin-Lowry syndrome
cM	centiMorgan
CNS	central nervous system
CREB	c-AMP responsive element binding protein
DFN3	X-linked sensorineural deafness type 3
DH	Dbl homology
ERK	extracellular signal-regulated kinase
EST	Expressed Sequence Tag
FISH	fluorescence in situ hybridization
FRAXE	fragile-X-E site in Xq28
GAP	GTPase activating protein
GDIα	GDP-dissociation inhibitor α
GDI1	gene encoding GDIα
GEF	guanine nucleotide exchange factor
GGT	geranylgeranyl transferase
GKD	glycerol kinase deficiency
HbH	hemoglobin H
IL	Interleukin
IL1R	interleukin-1 receptor
IL1RAcP	IL1 receptor accessory protein
IL1RAPL	IL1RAP-like
IQ	Intelligence Quotient
JNK	c-Jun amino-terminal kinase
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
Mb	megabases

MECP2	methyl-CpG binding protein 2
MR	Mental Retardation
MRX	nonspecific (or nonsyndromic) X-Linked Mental Retardation
MRXS	Syndromic X-Linked Mental Retardation
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
OMIM	Online Mendelian Inheritance in Man
OPHN1	oligophrenin-1
PAK3	p21-activating protein kinase 3
PH	pleckstrin homology
PIX	PAK-interacting exchange factor
PKA	protein kinase A
Rac1	Ras-related C3 botulinum toxin substrate 1
REP	Rab escort protein
RFLP	Restriction Fragment Length Polymorphism
RhoA	Ras homologous member A
RSK	Ribosomal S6-kinase
RTT	Rett syndrome
SSCP	single stranded conformation polymorphism
TM4SF	transmembrane-4 superfamily
TM4SF2	tetraspanin-2
TRD	transcriptional repression domain
XLMR	X-Linked Mental Retardation
XNP	X-linked nuclear protein
YAC	yeast artificial chromosome

Chapter 1

General introduction

1.1 MENTAL RETARDATION

1.1.1 Definition and prevalence of mental retardation

In our society, intelligence is thought to be very important for, if not determining, a person's success. Correct brain function permits the acquisition of skills that serve as hallmarks of developmental progress. Significant impairments of cognitive and adaptive performance are present in two to three percent of the human population, and are grouped under the general term mental retardation (MR). The most recent definition of MR, issued by the American Association on Mental Retardation[1] states: *Mental retardation refers to substantial limitations in present functioning. It is characterized by significantly subaverage intellectual functioning, existing concurrently with related limitations in two or more of the following applicable adaptive skill areas: communication, self-care, home living, social skills, community use, self-direction, health and safety, functional academics, leisure and work.* Furthermore, the mental manifestations must be present before the age of 18 years.

In this definition, MR is a typical human condition. For the affected individual, it implicates a limited capacity to learn and to achieve the goals that are thought to be essential to function independently in our society. For public health, MR is a common abnormality, that is widely distributed throughout the population and that imposes a costly and lifelong burden. For medicine and science, however, MR represents an aberration in the formation and/or function of the central nervous system that demands evaluation and explanation[2].

The most widely used system for classification of MR is based on severity as determined by performance on standardized intelligence tests. The different classes of MR vary in Intelligence Quotient (IQ), assuming a population mean of 100 and a standard deviation of 15. The criteria of the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10), coordinated by the World Health Organization (WHO), are summarized in Table 1.1.

Table 1.1. *Classification of mental retardation according to ICD-10 criteria*

Degree of mental retardation	Intelligence Quotient (IQ)	Age equivalent (years; months)
Borderline	70-85	
Mild	55-69	8;3-10;9
Moderate	40-54	5;7-8;2
Severe	25-39	3;2-5;6
Profound	<25	<3;2

Often a simplification of this classification into two large groups is used: severe MR, comprising IQ values below 50, and mild MR, including IQ values from 50 to 70. Using this classification, mild MR has an estimated prevalence of 2-3%[3], whereas 0.3% of the population is severely handicapped[4]. When MR is consistently associated with somatic, neurological, or behavioral manifestations, it is termed syndromic mental retardation. Mental retardation syndromes tend to have a single, specific, and identifiable heritable or

environmental cause. In the absence of consistent somatic, neurological, or behavioral manifestations, MR is termed nonsyndromic or nonspecific.

1.1.2 Etiology of mental retardation

As with other disorders of human development and functioning, mental retardation may result from genetic influences, environmental insults, or some combination of the two[5]. The disorder has a substantial genetic component and there may be a genetic cause in approximately half of the severely retarded patients[6]. The ranks of single-gene (Mendelian) disorders recognized to cause MR have grown steadily over the past half century. In august 2001, the McKusick catalogue of genes and phenotypes (Online Mendelian Inheritance in Man (OMIM); http://www3.ncbi.nlm.nih.gov/Omim) responds with 993 entries when queried with "mental retardation".

An excess of males has long been noted among persons with MR[7]. It is present in diverse populations worldwide, and among all degrees of mental deficiency. In the Netherlands, the male excess seems to be largest in the least severe type of mental impairment, i.e. specific learning disabilities, and gradually drops with increasing severity[8]. In most populations, the male excess is about 20%–40%. Greater mortality among females and hormonal contributions to the causation of MR, have been proposed as causes for the difference in incidence of MR among sexes, but these have never been proven. The different number of sex chromosomes between males and females is regarded to be primarily responsible for the excess of males with MR. Whereas a mutation in a gene on a male's single X-chromosome may cause MR, the same mutation in a female may be compensated for by the presence of a normal allele on her second X-chromosome. The mutation can also be silenced by preferential inactivation of the X-chromosome carrying the mutant allele. Accepting a male excess of 30%, it can be estimated that X-linked mental retardation is one of the most frequent causal categories of MR, equaling that of chromosomal aberrations. It is to be expected that most of the inherited forms of mental retardation are X-linked recessive. Dominant forms of mental retardation (autosomal and X-linked) might be found in sporadic cases or in families with mild mental retardation, but individuals with severe MR do generally not reproduce. Autosomal recessive forms of MR may be frequent, but for recognition of the inheritance pattern and disease causing gene(s) large families are required. Such pedigrees can be found in inbred populations. Furthermore, some frequent mutations with a high heterozygote frequency might be found in other populations. In contrast, MR segregating in an X-linked recessive manner is found in all populations, even if the carrier frequency is low. Males with a mutation in an X-linked gene will be affected, whereas heterozygous females will be non-manifesting carriers.

1.2 X-LINKED MENTAL RETARDATION

1.2.1 Definition and prevalence of X-linked mental retardation

X-linked mental retardation (XLMR) is that proportion of mental retardation showing the distinctive pattern of inheritance associated with the X-chromosome. The XLMR phenotype in a family can be detected when the transmission follows the general characteristics of X-linked recessive phenotypes[2]:

- *only males are affected*
- *sons of carrier females have a 50% risk of being affected*
- *daughters of carrier females have a 50% risk of being carriers*
- *male to male transmission does not occur*
- *all daughters of affected males will be carriers*
- *unaffected males cannot transmit the phenotype*
- *carrier females may have mild expression of the disease*

Using a 30% excess of males among persons with MR, and attributing all of the male excess to XLMR, one may calculate that XLMR makes up at least 13% of all mental retardation[2,9-13]:

Given that a = the percentage of females with non X-linked MR
b = the percentage of females with XLMR
c = the percentage of males with non X-linked MR
d = the percentage of males with XLMR

and $a+b+c+d = 100\%$
$c+d = 1.3\ (a+b)$
$a = c$

then $a+b+1.3\ (a+b) = 100\%$
$\leftrightarrow$ $2.3(a+b) = 100\%$
$\leftrightarrow$ $a+b = 43.5\%$ (all females with MR) and $c+d = 56.5\%$ (all males with MR)

Assuming that the number of females with XLMR is negligible ($b = 0$):

Then $a = 43.5\%$
$\leftrightarrow$ $c = 43.5\%$
$\leftrightarrow$ $d = 13\%$

Turner estimated, however, that possibly 10% of mild mental retardation in females, and 20-25% of all mental retardation in males is due to defects on the X-chromosome[14,15]. Indeed, these data fit in the above formula, if we use 10% of XLMR females:

a+b = 43.5% ↔ a = 33.5% and b = 10%
c+d = 56.5% ↔ c = 33.5% and d = 23%

XLMR males + females = 33%

To summarize, mutations in X-linked genes are the cause of 13% of MR, when disregarding the small amount of XLMR females. Since there are females with X-linked forms of MR, the contribution of X-linked genes is higher than 13% (see formula). In any case, X-linked genes are among the most common causes of MR. The percentage of 20-25% by Turner[14,15] is comparable with the portion of cases that can be attributed to chromosome aberrations (~12-15%)[16] and substantially exceeding the proportion that could be attributed to subtelomeric deletions (5%)[17,18] or autosomal genes (5-8%). However, studies of various cohorts of persons with MR have found X-linked genes responsible for 7.5% of cases or less[4,5,19-24]. A number of factors may contribute to the shortfall between the estimated prevalence and the identifiable prevalence of XLMR. Many XLMR entities may not have distinctive features which provide clues to clinical diagnosis. Furthermore, in some pedigrees X-linkage may not be immediately recognizable, e.g. if there is a new mutation or if there are affected females in the family[2].

1.2.2 Classification of X-linked mental retardation

XLMR can be divided into two broad categories: syndromic and nonsyndromic, also denoted nonspecific. In syndromic XLMR (or MRXS), somatic, neurological, behavioral, or metabolic abnormalities accompany the mental retardation in a recognizable pattern. One could expect that the genes involved in these syndromes have a general expression pattern in the body and that their function is not restricted to development of human intellectual learning. It is even possible that the gene is not expressed in the brain at all and that the mental retardation is a secondary event. In contrast, there is an increasing number of X-linked conditions in which MR is apparently the only clinical feature. These conditions are referred to as nonspecific XLMR (or MRX), and affected males have a normal physical appearance and no somatic, neurological, behavioral, or metabolic findings that distinguish them from non-affected male relatives or from other males[25]. MRX is clinically homogeneous but genetically heterogeneous. A recent update on XLMR genes was given by Chiurazzi *et al.*[26].

MRX has an estimated incidence of 1.8/1000 or 1 in 550 males[10]. This percentage is often wrongly quoted in the literature as the incidence of XLMR (i.e. MRXS and MRX combined). The prevalence of MR in the population is approximately 3%, and at least 13% of all MR in males is due to XLMR (see 1.2.1). The prevalence of XLMR in males is therefore approximately 0.39% or 1 in 256 males. This indicates that MRXS and MRX contribute equally to the incidence of mental retardation.

1.3 OUTLINE OF THE THESIS

With a population prevalence of 2-3%, mental handicaps constitute a serious medical and social problem in our society. The cause of the mental retardation is highly variable and involves both genetic and environmental factors. In spite of extensive medical investigations, the etiology of mental handicap remains unexplained in most patients. It has been estimated that at least 13% of all MR is caused by X-chromosomal gene defects (XLMR) (see 1.2.1). With a total of 100,000 newborn males per year, this means that approximately 400 males with XLMR are born each year in the Dutch population. Unraveling the genetic cause of MR will have important consequences for the prognosis, support, and possible treatment of the patients, and for genetic counseling of their relatives.

The study described in this thesis is part of a worldwide effort to search for genes that are important in the pathogenesis of nonspecific X-linked mental retardation (MRX). In order to achieve this, the primary method is accurate clinical examination and genetic linkage analysis with highly polymorphic markers in as many as possible MRX families. In the last few years, an international nomenclature system has been set up to collect these data and make them available to the scientific community. Since the publication of MRX1[27], a significant resource of mapped MRX families has been established. To date, in 76 MRX families the genetic defect has been linked to the X-chromosome, two of which are described in this thesis. MRX46 links to Xq25-q26 with a maximum lod score of 5.12 at θ=0 (*appendix 1*), and MRX65 links to the pericentromeric region with a maximum lod score of 3.64 at θ=0 (*appendix 2*).

Positional information about MRX genes, provided by linkage analysis, will pave the way for the final identification of disease-causing mutations in MRX candidate genes. However, the MRX linkage intervals are typically too large to successfully search for candidate genes. Moreover, it is not possible to narrow down these intervals because the individual MRX families can not be lumped together on the basis of their "common" phenotype. Since there may be more than 50 MRX genes, other strategies have to be followed in order to identify novel MRX genes. The high heterogeneity in MRX makes it necessary to screen more than 100 probands per candidate gene. In order to have access to such a large patient panel, the European XLMR consortium was established. Together with groups in Paris, Leuven, Berlin, and Tours, we collected approximately 200 well-characterized XLMR families.

In this thesis the identification of two (candidate) genes for MRX is described, as well as the subsequent mutation analysis in the material from the European XLMR consortium. In *appendix 3* the identification of a novel gene, *RSK4*, is described. This gene is the only known gene in the MRX critical interval in a contiguous gene syndrome in Xq21, which also involves deafness type 3 (DFN3) and choroideremia (CHM). Since no mutations have been found in the 200 consortium patients up to now, there is no definitive proof for the involvement of *RSK4* in MRX. The molecular analysis of a translocation breakpoint in

Xq26, the identification of the *ARHGEF6* gene and a mutation in family MRX46 is described in *appendix 4*.

Another way to identify MRX genes is to screen known genes in a large cohort of patients. Genes that cause a syndromic form of XLMR with a wide phenotypic spectrum are excellent candidate genes for MRX. An example is the *XNP* gene, that is involved in ATR-X syndrome and several other severe mental retardation syndromes. In *appendix 5* we describe a mutation in this gene in a family with mild mental retardation, in which only retrospectively mild phenotypic characteristics of ATR-X were found.

Chapter 2

Nonspecific X-linked mental retardation

2.1 NONSPECIFIC X-LINKED MENTAL RETARDATION

Before the availability of polymorphic molecular markers, no MRX entity had been mapped. A major advance in understanding MRX came in 1988 when Suthers *et al*.[27] employed restriction fragment length polymorphisms (RFLPs) to regionally map an MR condition to the pericentromeric region of the X-chromosome (Xp11.3-q12). The genetic defect was subsequently mapped in other MRX families, which revealed numerous non-overlapping linkage intervals on the X-chromosome. Thus, it became readily apparent that MRX is genetically heterogeneous. A convention, established in 1990, has assisted in the systematic study of MRX families[28]. Those families in which linkage analysis produced lod scores ≥ 2 were assigned MRX numbers. The subsequent development of microsatellite markers in the early 1990s has greatly accelerated the pace of regional mapping of these conditions[2]. To date, 76 MRX numbers have been assigned (Table 2.1). A number of MRX families remain outside this system, however, having been reported prior to adoption of the MRX convention or being of insufficient size or not informative enough to achieve a lod score ≥ 2[9,29-38]. Only in nine of the 76 MRX families a mutation has been found (indicated in italics in Table 2.1). Seven genes are involved in these families: *GDI1*[39,40], *OPHN1*[41], *PAK3*[42,43], *RSK2*[44], *IL1RAPL1*[45], *ARHGEF6* (*appendices 1 and 4*, this thesis) and *MECP2*[46]. Besides these genes, two more MRX genes have been described: *FMR2*[47,48] which is located next to a fragile site, and *TM4SF2* which is mutated in two small families in which no linkage analysis could be performed[49].

Table 2.1. *Mapping of MRX families. (Adapted from* http://xlmr.interfree.it/home.htm*)*

Name	Locus	Flanking markers	Gene	Reference
MRX1	Xp11.3-q12	DXS426-DXS1125		27,50,51
MRX2	Xp22.2-p21.3	DXS207-DXS1065		52,53
MRX3	Xq28-qter	DXS304-qter		54
MRX4	Xp11.23-q21.32	DXS255-DXYS1X		52,53
MRX5	Xp11.4-q21.31	OTC-DXS95		55
MRX6	Xq27	Not reported		56
MRX7	Xp11.23-q12	DXS1039-AR		57,58
MRX8	Xp11.3-q13.2	DXS1003-DXS1166		59,60
MRX9	Xp21.2-q12	DXS164-DXS453		61
MRX10	Xp21.3-p11.4	DXS28-DXS228		50,51
MRX11	Xp21.2-p11.4	DXS164-DXS7		50,51
MRX12	Xp21.2-p11.21	DXS1238-ALAS2		50,51
MRX13	Xp22.31-q22.3	DXS85-DXS456		50
MRX14	Xp11.3-q12	PFC-DXS135		62,51
MRX15	Xp21.2-p11.22	DXS164-DXS988		63
MRX16	Xq28		*MECP2*	46
MRX17	Xp11.23-q12	DXS255-DXS106		33,51
MRX18	Xp11.3-p11.23	DXS538-DXS1126		33
MRX19	Xp22.2-p22.1		*RSK2*	44
MRX20	Xp11.4-q21.33	DXS1068-DXS454		64
MRX21	Xp22.3-p11.4	Xpter-DXS7		65
MRX22	Xp21.1-q21.32	DXS84-DXSYS1X		66
MRX23	Xq22.3-q25	DXS1105-DXS425		67
MRX24	Xp22.32-p22.2	DXS278-DXS207		68
MRX25	Xq27.3-qter	DXS297-qter		69
MRX26	Xp11.4-q21.33	MAOB-DXS454		70
MRX27	Xq23-q26.3	DXS424-DXS102		71

Table 2.1 (continued).

Name	Locus	Flanking markers	Gene	Reference
MRX28	Xq28-qter	DXS1113-qter		[72]
MRX29	Xp22.13-p21.3	DXS989-DXS1234		[73]
MRX30	Xq22		*PAK3*	[42]
MRX31	Xp11.23-q13.3	DXS1126-DXS1124		[74]
MRX32	Xp22.2-p21.3	DXS1053-DXS992		[75]
MRX33	Xp22.13-p11.4	DXS365-MAOB		[72]
MRX34	Xp22.1-p21.3		*ILRAPL1*	[45]
MRX35	Xq22.1-q26.1	DXS178-HPRT		[76]
MRX36	Xp22.13-p21.1	DXS989-DMD		[37]
MRX37	Xp22.32-p22.31	DXS1223-DXS1224		[38]
MRX38	Xp22.13-p21.1	DXS1226-DXS1238		[77]
MRX39	Xp11	Translocations		[78]
MRX40	Xq21.1-q21.2	DXS233-CHM		[79]
MRX41	Xq28		*GDI1*	[40]
MRX42	Xq24-q25	not reported		[80]
MRX43	Xp22.31-p21.1	DXS987-DMD		[81]
MRX44	Xp11.3-p11.21	DXS1003-ALAS2		[81]
MRX45	Xp11.3-p11.21	DXS1003-ALAS2		[81]
MRX46	Xq26		*ARHGEF6*	*Appendices 1 and 4,* this thesis
MRX47	Xq22		*PAK3*	[43]
MRX48	Xq28		*GDI1*	[40]
MRX49	Xpter-p22.31	Xpter-DXS8022		[82]
MRX50	Xp21.1-p11.21	DXS8012-DXS991		[82]
MRX51	Xp21.1-p11.23	DXS8012-DXS1003		[83]
MRX52	Xp11.21-q21.33	ALAS2-DXS3		[81]
MRX53	Xq22.3-q25	DXS1210-DXS1047		[84]
MRX54	Xp22.13-p21.3	DXS989-DXS1218		[85]
MRX55	Xp11.4-q12	DXS1068-DXS1275		[86]
MRX56	Xp21.1-p11.21	Not reported		[80]
MRX57	Xq24-q25	Not reported		[80]
MRX58	Xp22.13-q12	DXS989-DXS1111		[87]
MRX59	Xp22.31-p21.1	DXS987-DMD		[88]
MRX60	Xq12		*OPHN1*	[41]
MRX61	Not reported	Not reported		
MRX62	Xq21.33-q25	DXS458-DXS737		Ronce *et al.*, unpublished data
MRX63	Xq21.33-q27.2	DXS990-DXS1227		Moraine *et al.,* unpublished data
MRX64	Xq28	DXS1113-qter		[89]
MRX65	Xp11.23-Xq21.33	DXS573-DXS990		*Appendix 2,* this thesis
MRX66	Not reported	Not reported		
MRX67	Not reported	Not reported		
MRX68	Xq21.33-q23	DXS8020-DXS1220		Frints *et al*., unpublished data
MRX69	Xp11.21-q22.1	DXS991-DXS178		Frints *et al.*, unpublished data
MRX70	Xq23-q25	DXS8063-DXS1047		[83,90]
MRX71	Xq24-Xq27.3	DXS1001-DXS8043		Frints *et al*., unpublished data
MRX72	Xq28	DXS1073-F8c		[91]
MRX73	Not reported	Not reported		
MRX74	Xp11.4-p11.3	DXS556-DXS1003		Ronce *et al.*, unpublished data
MRX75	Xq25-q26.2	DXS8098-DXS1062		[92]
MRX76	Xp22.31-p21.3	DXS987-DXS8039		Kleefstra *et al.*, unpublished data

Note: Localization of flanking markers according to the "Sixth International Workshop on Human X Chromosome Mapping 1995"[93] and http://www.gdb.org (for those not displayed in [93]). Therefore, the subchromosomal localization (locus) displayed in this table might slightly differ from that originally reported.

Figure 2.1 presents the linkage data from all reported MRX families with a lod score above two. Furthermore, the nine known MRX genes are indicated, as well as the *XNP* gene that was found mutated in a family in which only retrospectively some phenotypic characteristics were found (*appendix 5*, this thesis). Many of the published families have overlapping linkage intervals. Assuming that these families have allelic mutations, the X-chromosome comprises 8 exclusive linkage groups, indicating that at least 8 additional genes are involved in the 76 families reported. However, each of the reported MRX genes is involved only in one or two families, indicating that a linkage group is likely to contain more than one MRX gene. Hence, it is plausible that more than 50 MRX genes exist.

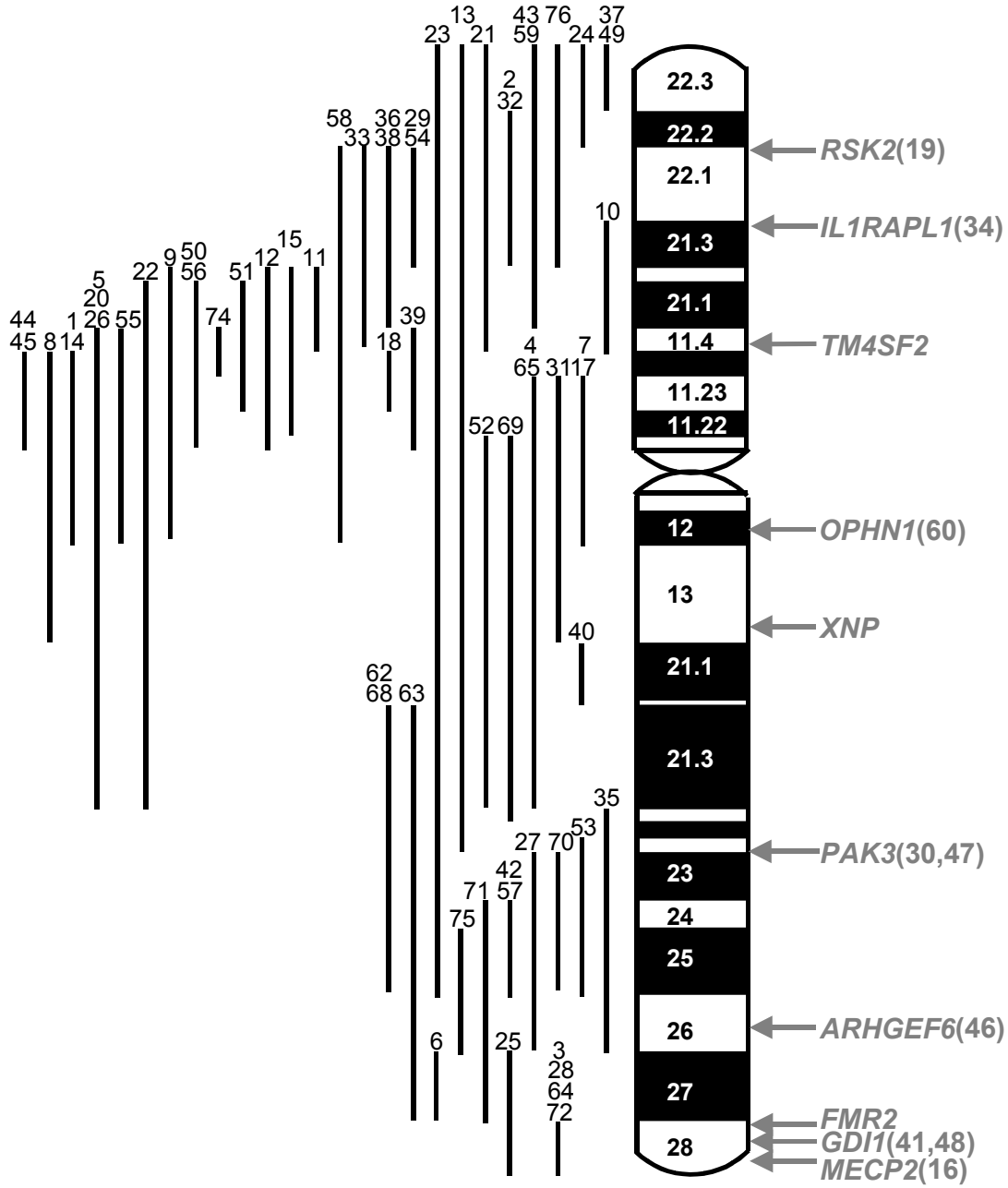

Figure 2.1. *Chromosomal distribution of MRX loci. Left: linkage intervals on the X-chromosome in MRX families with lod score >2. Right: MRX genes and the family numbers in which a mutation is identified.*

2.2 IDENTIFICATION OF MRX GENES

Complete analysis of individual large MRX families using a dense map of highly informative markers will allow, at best, a localization within a 10-15 megabases (Mb) interval. The complete X-chromosome is approximately 163 Mb and contains an estimated maximum of 1500 genes[94,95]. With a gene density of 7 to 11 genes per Mb, a linkage interval in an individual family therefore can contain more than 100 genes. In view of the high genetic heterogeneity, an overlap in localization between two MRX families cannot be taken as an indication that the same gene is implicated, and thus, cannot be used to obtain a more restricted localization. Linkage data can be very useful however, as soon as a candidate gene for MRX is identified. Probands of families with corresponding localization can be screened for mutations in these genes. Once the conditions for analysis of mutations have been defined for a given gene, they can be used systematically on a relatively large number of samples in a single experiment, in which also families without linkage data can be tested.

There are generally three ways to identify candidate genes for MRX:

1. Positional cloning

2. (Positional) candidate gene analysis

3. Mutation analysis of known MRXS genes

2.2.1 Positional cloning

Positional cloning is based on the chromosomal localization of the gene, i.e. the position of the gene in the human genome, without using any prior knowledge about the biological function of the gene product. Information about the position can be obtained through linkage analysis in affected families. The linkage intervals are generally too large to successfully clone the disease genes. The cloning and identification of MRX genes is greatly facilitated by the presence of fragile sites or cytogenetic aberrations, like translocations, inversions, and deletions. Especially translocations in mentally retarded patients provide an excellent opportunity to clone the X-chromosomal gene disrupted by the translocation. Also deletions encompassing a number of contiguous genes on the X-chromosome have been described, and in some instances the deletions are presumed to include genes that cause MRX. Microscopically visible male viable deletions of the X-chromosome are clustered in two regions, Xp22.3–Xp21, and Xq21. These regions also harbor microdeletions[96-101].

Positional cloning has been successful in the identification of five of the nine known MRX genes: *FMR2*, *OPHN1*, *TM4SF2*, *IL1RAPL1*, and *ARHGEF6*. In 1996, the *FMR2* gene, which was located next to the FRAXE fragile site in Xq28, was cloned by analysis of the DNA of two unrelated boys with submicroscopic deletions near FRAXE and mental retardation[47]. *FMR2* is the first MRX gene described. The molecular basis of FRAXE has been identified as the absence[47,48] or truncation[47] of the FMR2 protein. This is a consequence of either transcription silencing of the *FMR2* gene through methylation of the

FRAXE CpG island in subjects with the full mutation (> 200 CGG copies)[102], or deletion within the *FMR2* gene creating null alleles[47,103]. No *FMR2* point mutations have been identified so far.

Positional cloning of the X-chromosomal gene disrupted by an X-autosome translocation in a mentally retarded individual, and subsequent mutation analysis in MRX families has been successful in the identification of three MRX genes. Billuart *et al*.[41] identified a novel gene with 25 exons, *OPHN1* or *oligophrenin-1*, that was spanning the breakpoint in Xq12. A single base-pair (bp) deletion, resulting in a frameshift, was detected in family MRX60. The *TM4SF2* gene at Xp11.4 is inactivated by the X-chromosomal breakpoint of a balanced translocation t(X;2)(p11.4;p21.3) in a female patient with MR and autistic behavior[49]. Subsequently, in two of 33 MRX families mutations were found (missense and nonsense, respectively). Molecular analysis of a reciprocal X/21 translocation in a male with mental retardation showed that the *ARHGEF6* gene in Xq26 was disrupted by the rearrangement. Mutation screening of 119 patients with nonspecific mental retardation revealed a mutation in MRX46 (*appendices 1 and 4*, this thesis).

Mapping of four contiguous gene deletion syndromes exhibiting MR with only adrenal hypoplasia or with adrenal hypoplasia and glycerol kinase deficiency (GKD), and three deletions without MR but with either adrenal hypoplasia or adrenal hypoplasia and GKD, revealed a locus for MRX in the Xp22.1-p21.3 region[104]. This locus was confirmed by the detection of two inherited overlapping microdeletions in Xp22.1-p21.3 associated exclusively with MRX[100,101,105]. In one of these families, MRX34, the deletion appeared to span only 350 kb, and comprises three of the eleven exons of a novel gene. This gene shows homology to the Interleukin (IL)-1 receptor accessory protein (*IL1RAP*), and is named IL1RAPL1 (for IL1RAP-like-1)[45]. In a small MRX family, a nonsense mutation was detected, leading to a barely detectable amount of *IL1RAPL1* transcript.

Positional cloning strategies have not always been successful in the identification of MRX genes. In several cases, a gene has been cloned that is involved in a chromosomal aberration, but subsequent mutation analysis in a large panel of MRX families failed to prove the causal involvement of the gene in MRX. Examples are *DXS6673E*[106] and *GRIA3*[107], both disrupted by a translocation, and *VCX-A*[108] and *RSK4* (*appendix 3*, this thesis), which are located in the MRX critical interval in contiguous gene deletion syndromes. In several cases no gene was found to be disrupted by the translocation or inversion[109]. The fact that in many cases no MRX gene could be identified, indicates that (in case of a translocation) the autosomal breakpoint could contain a gene for MR, or that large rearrangements per se can be causative for MR. Since translocation breakpoints can affect the expression of a gene several hundreds of kilobases away[110], a position effect can be an important reason for the lack of gene identification by positional cloning. The position effect can be caused by the dissociation of a regulatory element from the gene, juxtaposition of the gene with an enhancer element from another gene, or integration of a gene into a region with another chromatin structure[110]. A well known example of a

position effect leading to disease is the chromosomal rearrangement involving *POU3F4* in X-linked deafness type 3 (DFN3). Mutations have been found within the gene[111], but micro-deletions that overlap in an 8 kb fragment located 900 kb upstream of the *POU3F4* gene have also been described[111-113]. This hotspot for microdeletions is thought to disconnect the *POU3F4* gene from a regulatory element[112,114]. Finally, in all cases in which no gene could be identified by positional cloning, it cannot be ruled out that there is no correlation between the mental retardation and the observed chromosomal aberration.

2.2.2 (Positional) candidate gene analysis

Due to the Human Genome Project, the number of cloned genes on the X-chromosome has increased rapidly over the last few years. Genes that are known to play a role in brain development are functional candidate genes for mental retardation. Strictly taken, functional candidate gene analysis is rarely performed nowadays, because positional information on linkage intervals and precise localization of genes is almost always available. In a positional candidate gene approach, mutation analysis of functional candidate genes will be focused on families with linkage intervals encompassing the candidate gene, or conversely, the most promising candidate genes in a known linkage interval will be analyzed[115]. Two of the nine known MRX genes have been found by a positional candidate gene approach: *GDI1* and *PAK3*.

D'Adamo *et al*.[40] searched for mutations in genes that map to Xq28 and are predominantly expressed in the brain. One gene that met these criteria was *GDI1*, which encodes GDP-dissociation inhibitor α (GDIα). GDIα forms complexes preferentially with RAB3A, which is crucially important for synaptic neurotransmitter release[116-120]. Because *GDI1* was such a promising candidate gene for MRX, D'Adamo *et al*.[40] set out for mutation analysis of this gene, and identified a missense and a nonsense mutation in families MRX41 and MRX48, respectively. Subsequently, a missense mutation in a family with nonspecific X-linked semidominant MR was reported by another group[39].

In the search for the gene involved in the neural migration disorder X-linked lissencephaly (X-Lis)[121,122], a gene that was highly expressed in brain (cerebral cortex and hippocampus) was identified. This gene, denoted *PAK3*, was disregarded as the X-Lis gene, but was still a strong candidate for mental defects mapping to Xq24. Following a positional candidate disease analysis, mutations in the *PAK3* gene have been found in MRX30[42] and MRX47[43].

2.2.3 Mutation analysis of known MRXS genes

The difference between syndromic and nonspecific MR is obvious for clinical and diagnostic purposes. However, in the analysis of common genetic disorders, one should keep in mind that even the same mutation in a single gene can produce a remarkably wide range of associated clinical phenotypes. It is therefore reasonable to expect that a proportion of patients with mild or severe MRX will have mutations in genes already

known to cause syndromic forms of MR. In several X-linked mental retardation syndromes a broad phenotypic spectrum has been observed. Mutations in the Ribosomal S6-kinase 2 gene (*RSK2*) have been described in Coffin-Lowry syndrome (CLS; MIM 303600), which is characterized by mental retardation with a peculiar pugilistic nose, large ears, tapered fingers and drumstick terminal phalanges. A variable phenotypic expression pattern was observed in CLS and a mutation has also been described in a family in which the phenotype was unusual in that the degree of mental retardation and dysmorphic features was milder[123]. The finding of a missense mutation in family MRX19[44], made *RSK2* the first gene involved in syndromic and nonspecific XLMR, and paved the way for the analysis of other MRXS genes in families with MRX.

Recently mutations in the *MECP2* gene, encoding methyl-CpG binding protein 2, have been identified in males with nonspecific MR[124] and in MRX16[46]. Until then, mutations were only found in females with Rett syndrome (RTT; MIM 312750), a severe X-linked dominant neurodegenerative syndrome. Mutations in males were considered to be lethal[125]. Another example of extreme clinical heterogeneity is the X-linked mental retardation syndrome with α-thalassemia (ATR-X; MIM 301040) in which mutations have been found in X-linked nuclear protein (*XNP*)[126]. Mutations in the *XNP* gene have subsequently been reported in families affected with Juberg-Marsidi syndrome (MIM 309590)[127], Carpenter-Waziri syndrome[128], severe mental retardation with spastic paraplegia[129], Holmes-Gang syndrome[130], and Smith-Fineman-Meyers syndrome (MIM 309580)[131].

Recently, an *XNP* null mutation was found in a family in which two patients had moderate to profound MR and the typical facial characteristic features of ATR-X syndrome. Two other patients from the same family and with the same *XNP* mutation had mild MR and epilepsy but did not have the characteristic facial dysmorphisms[132]. Subsequent mutation screening of a large number of patients with several phenotypes including MRX, revealed a missense mutation in a family diagnosed with nonspecific XLMR (*appendix 5*, this thesis). The mental retardation was borderline to moderate. Only with hindsight and upon careful scrutiny of the available data, childhood hypotonia was seen in some patients. Because of the molecular findings, HbH inclusion bodies were sought for and found. These are characteristic for ATR-X syndrome.

These data suggest that the involvement of a particular gene in both MRXS and MRX is possible, and that future studies will reveal additional examples. Other candidates comprise the genes for XLMR with aggressive behavior (*MAOA*)[133], Aarskog-Scott syndrome (*FGD1*)[134], and Norrie disease (*NDP*)[135].

Chapter 3

MRX genes and their function

3.1 DEVELOPMENT OF MEMORY

3.1.1 Axon outgrowth

Once a neuron has migrated to its final position, it begins to extend an axon at its growing tip by means of a specialized structure, called the growth cone. Growth cones appear as an enlargement of the shaft of the axon. Several finger-like extensions, called filopodia, project from the growth cone[136]. Filopodia continually extend and retract. In between the filopodia are thin membranes called lamellipodia[137], which are also motile and give the growth cone its characteristic ruffled appearance (Figure 3.1). Both the lamellipodia and filopodia contain a high density of actin filaments[138]. Microtubules are found mainly in the cell body, the shaft of the neuron and the central region of the growth cone[139]. Growth cone motility is thought result from actin polymerization. Three members of the Rho subfamily of small GTP-binding proteins (Rho, Rac, and Cdc42) as well as Ras have been shown to regulate the actin-based cytoskeleton in fibroblasts (reviewed by Nobes and Hall[140]) and may have similar roles in neuronal growth cones. Studies in both fibroblast and neuronal cell types have identified the following pathway: activation of Cdc42 leads to the formation of filopodia and to the stimulation of Rac. Ras can also activate Rac, which in turn gives rise to the formation of lamellipodia and the activation of Rho[141]. In fibroblasts, active Rho induces the formation of actin-dominated stress fibers (axial bundles of F-actin underlying the cell bodies) and focal adhesions (multimolecular complexes associated with the actin cytoskeleton that contain a number of structural proteins and proteins involved in signal transduction). In neurons, which do not have stress fibers and focal adhesions, Rho is thought to regulate neurite retraction (reviewed by Vancura and Jay[142]) (Figure 3.2).

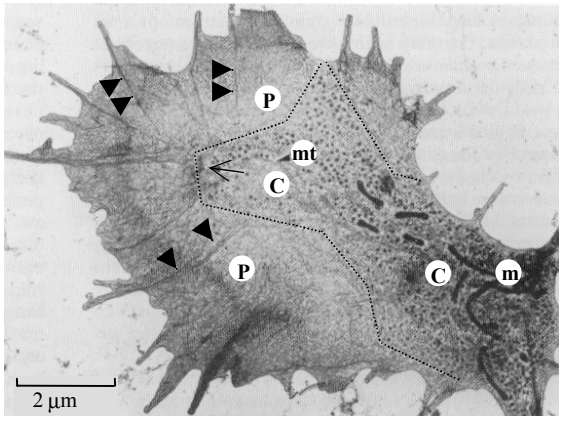

Figure 3.1. *Whole-mount electron micrograph of a growth cone in vitro. Two distinct cytoplasmic domains are present. The approximate boundary between the two domains is shown by the dotted line. The central (**C**) domain contains the microtubules (**mt**), mitochondria (**m**), and dense-core vesicles. The peripheral domain (**P**) contains bundles of actin microfilaments (arrowheads) that form the core of the majority of filopodia. (From Bridgman and Daily[143]).*

Axon growth requires an enormous investment on the part of the cell, as it must construct and extend a neurite process many times longer than the cell body. This requires transport of material through the growing axon as most of the proteins and membranes that comprise the axon are synthesized in the neuronal cell body. Members of the Rab subfamily of Ras-related small GTP binding proteins are key regulators of vesicular transport (Figure 3.2).

The outgrowth of neurons is always in a certain direction, mediated by the growth cones itself. This so called axon guidance is influenced by the contacts the filopodia make with other cells and with the extracellular matrix. In general, the growth cone moves in the direction where its filopodia make the most stable contacts. In addition, diffusible substances can bind to receptors on the growth cone surface, and so influence its direction of migration. Some of these cues promote axon extension (attractive cues), whereas others inhibit it by causing growth cone collapse (repulsive cues)[144-146]. Some cues act at long range and others at short range, thus giving four ways in which the growth cone can be guided.

Long-range attraction involves diffusible chemo-attractants released from the target cell, whereas short-range guidance is mediated by contact-dependent mechanisms involving molecules bound to other cells or to the extracellular matrix. The distinction between long-range and short-range guidance is mitigated by the finding that distant targets (long-range) are reached by making use of "stepping stones" or "guide posts": localized regions to which the growth cone moves when its filopodia make contact.

Many of the glycoproteins involved in neural adhesion, like NCAM, cadherins and integrins, contain extracellular immunoglobulin motifs. In addition, a variety of receptor tyrosine kinases in the axon plasma membrane can regulate axon outgrowth in response to extracellular signals.

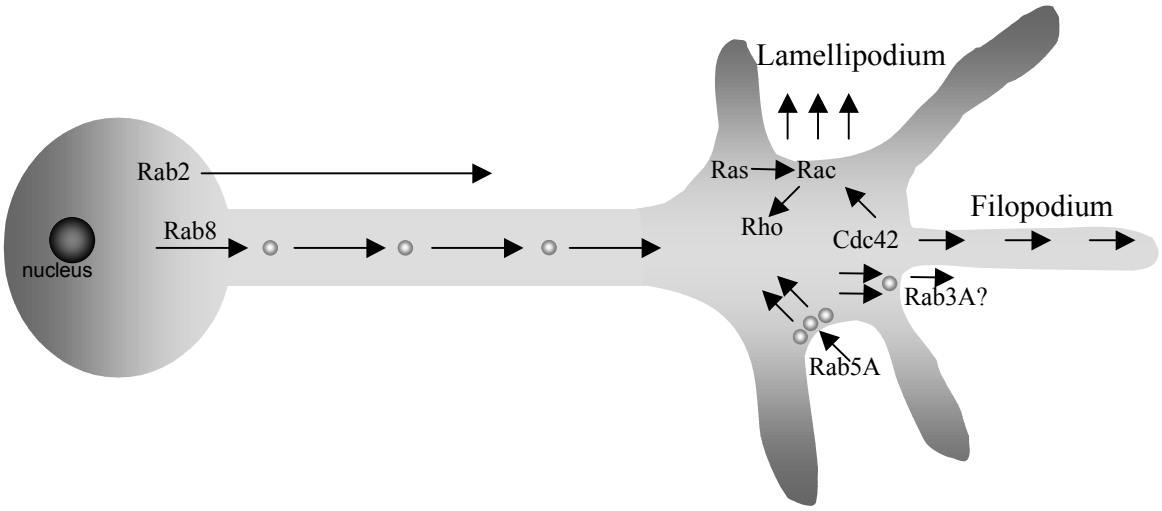

Figure 3.2. *Proposed roles for Rho GTPases in axon growth. Activation of Cdc42 leads to the formation of filopodia and to the stimulation of Rac. Ras can also activate Rac, which in turn gives rise to the formation of lamellipodia and the activation of Rho. Active Rho then causes neurite retraction. Various Rab family members (namely Rab2, 3A,5A, and 8) participate in different steps of vesicular transport or endocytosis. Activation of Rab2 results in increased spreading, adhesion, and neurite outgrowth, by mechanisms that remain unclear. Although Rab3A participates in synaptic vesicle fusion in mature neurons, the protein's role in endocytosis in immature neurons is equivocal. Rab5A is implicated as a regulator of endocytosis, whereas Rab8 is a key regulator of anterograde vesicle traffic in differentiating neurons. (Adapted from Vancura and Jay[142])*

3.1.2 Formation and refinement of synaptic connections

When axons reach their targets, specialized connections, called synapses, are made. These are essential for signaling between the neurons and their target cells. In the brain, the axon of one neuron makes contact with a dendrite from another neuron. Dendrites are formed in the same way as axons (via filopodia) and they extend from the cell body of the neuron. Rho affects the number of dendrites and the degree of branching[146], and thereby strongly determines the ability of the neuron to form synaptic connections. Contact between a presynaptic and a postsynaptic neuron is important for signaling, as well as for survival of the presynaptic neuron. If an axon does not reach its target, the neuron often dies. The survival of neurons depends on neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which are produced by the target cell and for which the neurons compete. The receptors for the neurotrophins are receptor tyrosine kinases. Different types of neurons require different neurotrophins for their survival, and the requirement for certain neurotrophins also changes during development.

For proper brain function, the initial contacts between the growth cone of the neuron and its target cell must be refined and stabilized. The process of stabilization involves the assembly of specialized structures that permit the release of synaptic transmitter from the nerve terminal and its efficient reception by the target cell. The presynaptic terminal regulates the number and organization of transmitter receptors and other molecules on the postsynaptic membrane. This process is best studied in the peripheral nervous system, but it is probably controlled in similar ways in the central nervous system. A primitive form of synaptic transmission exists from the moment the axon reaches the (muscle) target. First, the presynaptic axon is capable of releasing its neurotransmitter, acetylcholine (ACh), before it makes contact with its postsynaptic target cell. Second, the postsynaptic muscle membrane is capable of responding before it is contacted, via the presence of nicotinic ACh receptors on its membrane. Subsequently, the distribution of the ACh receptors changes and new receptors are formed, which ultimately leads to a cluster of ACh receptors at the synaptic site. The synapses are further stabilized by other molecules in the postsynaptic basal lamina, to ensure that the ACh receptors remain at the site of the regenerating synapse. Recognition of the basal lamina by the ingrowing nerve terminal may depend on specific glycoproteins found primarily at synaptic sites. In neuromuscular synapses, s-laminin is such a molecule[147].

Change in the strength of synapses is often referred to as “synaptic plasticity”, and changes in the strength of thousands of synapses in a neuronal network ultimately create human memory[148]. Memory storage in the mammalian brain is thought to be mediated by long-term potentiation (LTP) in the synaptic connections in the hippocampus[149]. The synapses in the hippocampus are extremely plastic, and a brief high frequency train of action potentials produces a long lasting increase in synaptic strength (LTP). The collateral axons in the hippocampus use glutamate as their transmitter. Glutamate produces LTP by

acting on at least two types of receptors in the dendritic spines: N-methyl-D-aspartate- (NMDA)- and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors[150]. The AMPA receptors mediate basal synaptic transmission, because the ion channel associated with the NMDA receptor is blocked by Mg^{2+} at the resting potential (voltage dependent block). Upon depolarization and binding of sufficient glutamate, the NMDA receptor is unblocked and activated. NMDA channels are permeable to Ca^{2+}. The induction of LTP depends on postsynaptic depolarization, the influx of calcium, and the subsequent activation of second messenger kinases in the postsynaptic cell. LTP has both short-term and long-term phases. Short-term (minutes to hours) changes create a transient memory, that does not require protein synthesis. For long-term changes the memory must be stabilized through gene transcription and synthesis of new synaptic proteins[151,152]. This requires the conversion of ATP to the second messenger cAMP by adenylyl cyclase. cAMP in turn activates protein kinase A (PKA). For long-term changes PKA must translocate to the nucleus, where it phosphorylates and activates a protein called cAMP response element-binding protein (CREB). CREB, a nuclear transcription factor, binds to specific cAMP response element (CRE) sequences on DNA and regulates the transcription of specific genes, including immediate early genes (i.e. needed for DNA replication and recombination) such as c-FOS[153].

3.2 FUNCTION OF MRX GENES

Specific abnormalities of dendrites have been found in some children with severe mental retardation and without other evidence of cortical malformation[154]. The anomalies include truncation of dendrites, simplification of dendritic arborization, malformed dendritic spines, and cytoskeletal disturbances[155]. These abnormalities have been described for Fragile X syndrome[156,157] and other developmental disabilities associated with MR, including Down syndrome[158-160]. Such abnormalities in brain microstructure could impair the ability of the brain to adequately process and store information, resulting in MR. It can be expected that some of the genes causative for MRX actually encode proteins that are required for axon outgrowth and/or the establishment and stabilization of neural connections. Indeed, the products of some of the MRX genes appear to be directly involved in these processes.

Currently, there are seven genes identified with mutations exclusively in MRX families, and two genes involved both in MRXS and MRX. Furthermore, the *XNP* gene is involved in clinically highly variable mental retardation syndromes, ranging from severe forms of ATR-X syndrome to apparent MRX. *GDI1* is involved in vesicle trafficking necessary for axon outgrowth. *OPHN1*, *ARHGEF6*, and *PAK3* regulate the Rho GTPases that drive axon outgrowth, and that activate a downstream MAPK pathway. *TM4SF2*, *RSK2*, and probably *FMR2* play a role in this pathway, and have a function in axon guidance and the stabilization of synapses that ultimately leads to memory formation. The

function of *IL1RAPL1* is unknown, but it might play a role in synaptic neurotransmitter release. *MECP2* and *XNP* are thought to be regulators of epigenetic gene silencing. In the following paragraph the proposed function(s) of the MRX(S)-gene products is discussed.

GDI1

Movement of proteins along the exocytic and endocytic pathways of the eukaryotic cell occurs by small vesicular carriers that mediate selective transport[161]. Essential for this process are a large group of prenylated small GTPases encoded by the Rab gene family. Rab proteins exist in both inactive GDP-bound and active GTP-bound forms. Conversion between the two forms is promoted by factors that either accelerate guanine-nucleotide exchange (GEF), or stimulate guanine-nucleotide hydrolysis (GTPase activating proteins; GAP)[162].

Rab proteins bind to the peripheral membrane of synaptic vesicles and regulate the fusion events that underlie endocytosis and exocytosis. The traffic-directing function of Rab proteins depends upon their anchoring to vesicle membranes, and this in turn depends upon the post-translational addition of geranylgeranyl lipids to their carboxyl termini[163]. This addition is carried out by Rab geranylgeranyl transferase (GGT)[164], a multicomponent enzyme consisting of a catalytic component B, and a Rab escort protein (REP; component A)[165]. The binding of REP to Rab further provides an "escort" service to mediate membrane docking of the Rab protein. Following membrane fusion and inactivation by GAP, a GDP-dissociation inhibitor (GDI) extracts the GDP-bound Rab from the membrane to the cytosolic reservoir for re-use[166,167]. In the subsequent step of the pathway (Figure 3.3), GDI is released to the cytosol and Rab can again be converted to the GTP-bound form by GEF.

In mammals, three different GDI isoforms (GDIα, -β, and -γ) have been described that interact with more than 30 different Rab proteins and participate in different fusion processes[168,169]. In the mammalian brain, the most abundant form is GDIα[167], which complexes preferentially with Rab3A. Rab3A is crucially important for synaptic neurotransmitter release[119,120]. In humans GDIα is encoded by the *GDI1* gene in Xq28[166]. It shows homology to the *REP1* gene in Xq21, which is involved in choroideremia and is an escort protein for several Rabs, including Rab1A, Rab3A, and Rab27[170-172]. Two missense mutations and a nonsense mutation in *GDI1* were found to be associated with MRX[39,40]. These mutations suggest that the MRX is caused by either the abolished synthesis of GDIα (nonsense mutation in MRX48) or by a reduced ability to form a complex with the GDP-bound form of Rab (missense mutations in MRX41 and R). Indeed for the L92P mutation in MRX41 a 6-fold decrease in the affinity between Rab3A and GDIα was demonstrated[40]. All three mutations will lead to a significant decrease in the pool of Rab proteins available for synaptic vesicle cycling. Although it was suggested first that the mental retardation in families with a *GDI1* mutation is the result of a defect in synaptic activity, there is evidence that GDIα is also involved in vesicle transport necessary for axon outgrowth. Mouse

studies revealed that Gdiα is expressed and upregulated early in brain development, before any synaptic activity is detectable[40]. Moreover, rat hippocampal neurons[40] and PC12 cells[173] treated with Gdi1 antisense oligonucleotides exhibited inhibition of axonal growth. Therefore, it seems likely that GDIα expression is essential for outgrowth, and not for differentiation, of axons during brain development. This role for GDIα in neural development is supported by the results obtained in Gdiα-deficient mice. These mice are viable and fertile, and showed no morphological abnormalities in their brains[174]. The Gdiα-deficient mice showed normal steady-state levels of synaptic proteins, including Rab3A, and a sharp increase in facilitation of excitatory hippocampal neurotransmission[174]. Behavioral analysis and morphological studies on brain microstructure in the GDIα-deficient mice may yield better insight in the pathology of XLMR.

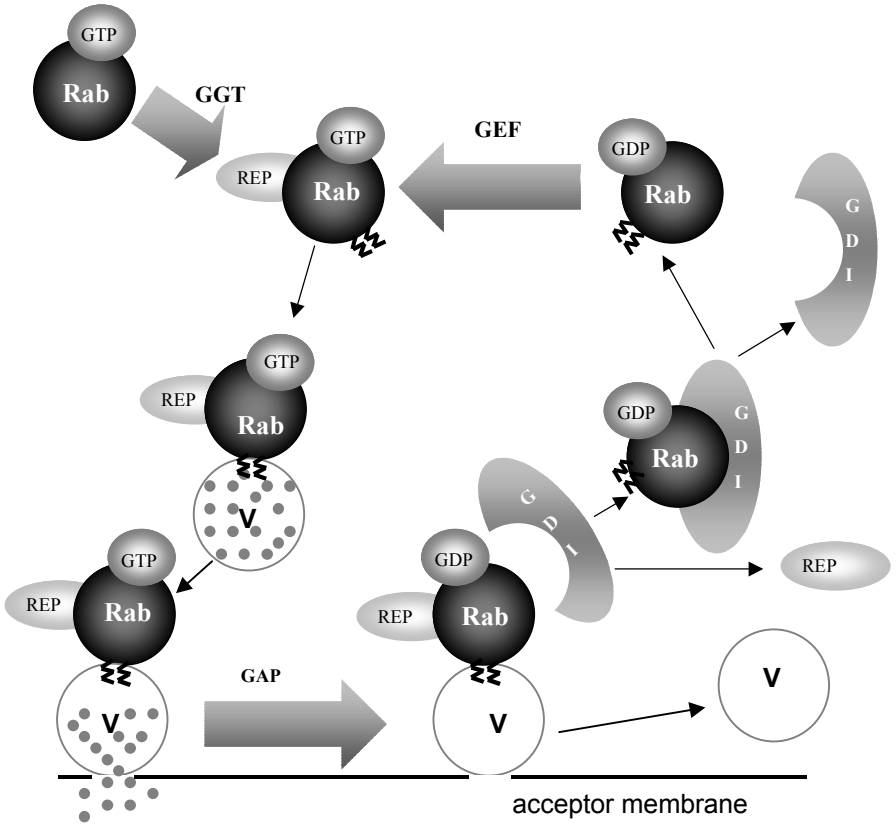

Figure 3.3. *Synaptic vesicle (**V**) transport, mediated the small Rab GTPases. Rab geranylgeranyl transferase (GGT) adds two geranylgeranyl tails to the Rab proteins, necessary for anchoring to the vesicle membrane. One of the components of GGT, REP, provides an escort service to facilitate membrane docking. Conversion between the GTP- and GDP-bound form is mediated by GEFs and GAPs. GDI retrieves the GDP-bound Rab from the membrane.*

OPHN1

Billuart *et al*.[41] characterized the X-chromosomal breakpoint of a t(X;12)[175] in a female MRX patient. A novel gene with 25 exons, *OPHN1* or *oligophrenin-1*, was spanning the breakpoint in Xq12. A frameshift mutation, leading to a premature stopcodon, has been identified in family MRX60[41,176]. *OPHN1* encodes a protein that contains a GTPase activation domain (GAP) for Rho GTPases. Like all members of the Ras superfamily, the activity of the Rho GTPases is determined by the ratio of their GTP/GDP-bound forms in the cell[162], which is regulated by the opposing effects of GEFs and GAPs. Despite their name, small GTPases hardly have any intrinsic GTPase activity. GAPs increase the intrinsic rate of hydrolysis of bound GTP. GEFs enhance the exchange of bound GDP for GTP. In addition, the Rho-like GTPases are regulated further by GDIs, which can keep the Rho proteins in their GDP-bound state (Figure 3.4).

The best-studied Rho GTPases are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42), often referred to as Rho, Rac and Cdc42. In addition to their downstream effects on the actin cytoskeleton, Rho family members affect each other's activity, and a signal-transduction pathway from Cdc42 to Rac to Rho has been proposed[141]. Several proteins that exhibit GAP activity for Rho GTPases have been identified in mammalian cells (reviewed by Van Aelst and D'Souza-Schorey[177]). These proteins all share a related 140 amino acids GAP domain, but the specificity for members of the Rho subfamily varies with each GAP protein. In addition to accelerating the hydrolysis of GTP, Rho GAPs may mediate other downstream functions of the Rho proteins in mammalian systems as an effector[178-181]. The link between different Rho GTPase cascades might be established by complex formation of different GAPs[182,183].

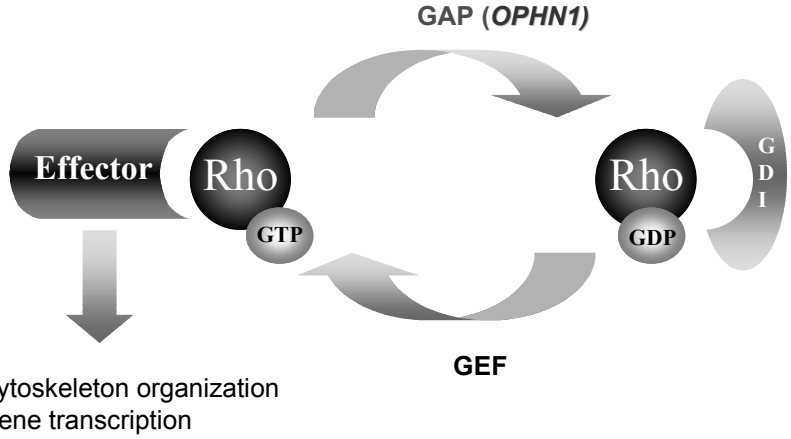

Figure 3.4. *The switch between the active (GTP-bound) and inactive (GDP-bound) form of Rho is mediated by GTP-activating proteins (GAP) and guanine nucleotide exchange factors (GEF). OPHN1 acts as a GAP for the Rho GTPases Rho, Rac, and Cdc42, and is therefore indirectly involved in the organization of the actin cytoskeleton and in gene transcription.*

Oligophrenin-1 is highly expressed in fetal and adult brain and in several other tissues, and shows in vitro GAP activity for the Rho GTPases Rho, Rac and Cdc42, but not for other small GTPases tested[41]. *OPHN1* is expressed in primary cultures of neuronal and

glial cells (Chelly *et al*., pers. comm.), which corresponds with the recent finding of overexpressed *oligophrenin-1* in human glial tumors[184]. Both the disruption of *OPHN1* by the translocation breakpoint and the frameshift mutation in MRX60 predict a loss of function of the oligophrenin protein, which may result in constitutively active Rho GTPases. This may lead, either directly or indirectly, to aberrant formation of neuronal processes as was shown in mice transgenic for an activated form or Rac[144] and in cortical cells transfected with constitutively active forms of Rho, Rac, or Cdc42[146].

PAK3

PAKs (p21-activated kinases) are effectors of the Rho-like family of small (21 kDa) GTP-binding proteins, hence their name. Mammalian tissues contain at least four PAK isoforms: PAK1 (Pakα), which is highly expressed in brain, muscle and spleen[185-187]; PAK2 (Pakγ), with ubiquitous tissue distribution[186,188,189]; PAK3 (Pakβ), which is highly enriched in brain, but with a cell-type distribution that differs from that of PAK1[180,190]; and PAK4, most highly expressed in prostate, testis and colon[191]. The PAKs contain at least four recognizable sequence elements: two-to-four proline-rich regions that may recognize Src-homology 3 (SH3) domains; a domain referred to by different authors as p21-binding domain (PDB), GTPase binding domain (GBD) or Cdc42/Rac1 interactive binding domain (CRIB); an acidic region; and a C-terminal serine/threonine protein kinase domain (reviewed by Sells and Chernoff[192]).

The PDB/GBD/CRIB domain of all mammalian PAKs is able to bind to the small Rho GTPases Rac1 and/or Cdc42. Upon this binding, the PAKs autophosphorylate at several serine and threonine residues and acquire increased kinase activity towards exogenous substrates. PAK kinase activity is essential for the Rho-mediated disassembly of focal adhesions and actin polymerization in fibroblasts[185,193-195]. The recruitment of PAK to the focal complexes can take place via binding of its N-terminal proline-rich domain to the SH3-domain of PAK interacting exchange factor (PIX)[196]. Since PIX is also a GEF for Rac and Cdc42, a potential feedback loop is created in which PAK acts downstream of Rac and Cdc42 as an effector, but also upstream via its binding to PIX[196]. The PAK/PIX interaction allows a signal transduction from Cdc42 to Rac[197]. Via the activation of Rac and Cdc42, PAK is involved in the formation of lamellipodia and filopodia[198], but these functions do not require PAK kinase activity[199,200]. The kinase activity of PAK is required in the nucleus for phosphorylation of MEK1 and RAF1[201-203], and the control of the Jun N-terminal kinase MAPK pathway (Figure 3.5).

PAK3, via its N-terminal non-catalytic domain, acts upstream of Rac mediating lamellipodia formation through interaction with βPIX[197]. The interaction with αPIX or βPIX stimulates the activity of PAK3[204]. Mutations in the *PAK3* gene have been found in MRX30[42] and MRX47[43]. The MRX30 mutation introduces a premature stop codon in the kinase domain of the protein and produces a stable truncated protein lacking kinase activity and an autophosphorylation site, although the truncated protein can still bind

activated Rac and Cdc42[42]. The missense mutation in MRX47 is located in a polybasic region upstream of the PDB/GBD/CRIB domain and predicts a reduced GTPase binding and stimulation of PAK activity[43]. Thus, both mutations have an effect on the kinase-dependent function of PAK3. Both mutations leave the SH3-binding motif (and therefore the possibility of binding to PIX) intact. This means that the kinase-independent activities of PAK3 are not blocked by the mutation. The mental retardation in these families might therefore be caused by a defect in activation of MAPK signaling and thus activation of transcription and synaptic plasticity.

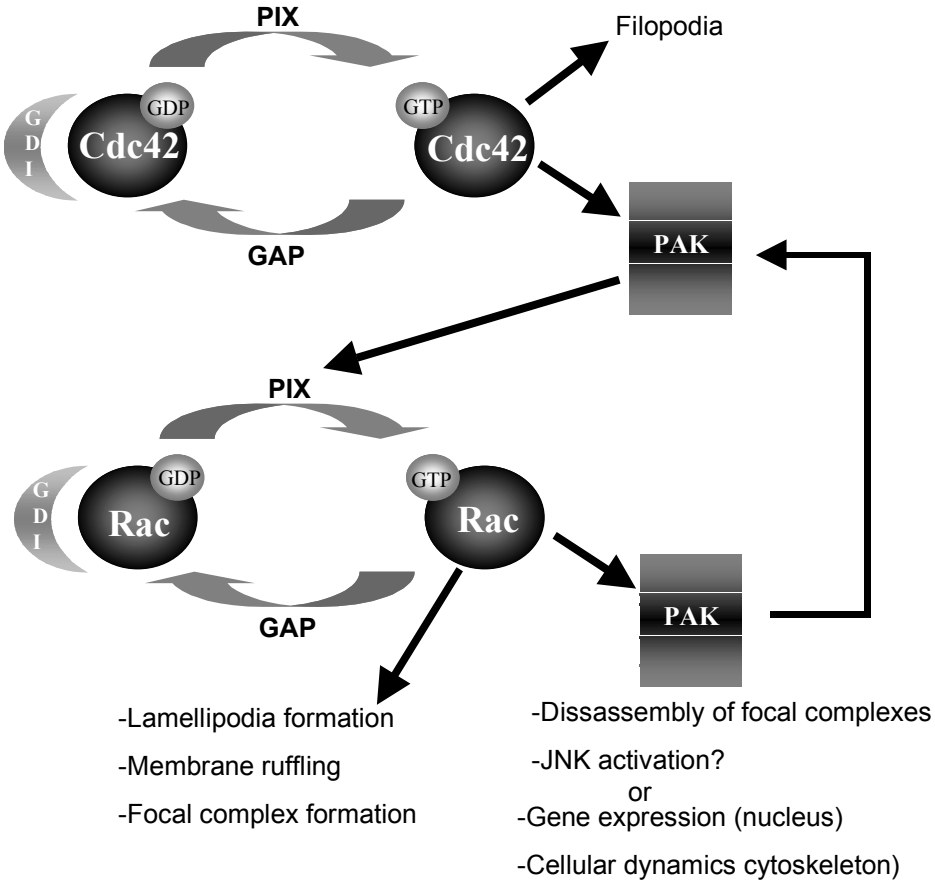

Figure 3.5. *PIX proteins act as guanine nucleotide exchange factors (GEFs) for the Rho GTPases Cdc42 and Rac. Furthermore, they can bind to PAK kinases, thereby linking Cdc42 to Rac. PAK has a function upstream of Rac mediating morphological effects through interaction with PIX. At the same time this does not rule out kinase dependent actions as a downstream effector of Rac. Cdc42 and Rac are involved in the formation of filopodia and lamellipodia, respectively. (Adapted from Obermeier et al.[197], and Van Aelst and D'Souza-Schorey[177])*

ARHGEF6

Manser *et al.*[196] identified two PAK-binding proteins containing Src-homology 3 (SH3), Dbl-homology (DH) and pleckstrin-homology (PH) domains, which they named αPIX and βPIX (for: PAK-interacting exchange factors) (see above text for PAK3). αPIX also contains a region homologous to the calponin-related domains (CH) of Vav and IQGAP, necessary for actin binding[205]. Binding of proline-rich regions of PAK kinases takes place via the SH3-domain of PIX[196]. The PH domain is a lipid-binding surface and

probably also a protein-protein interaction surface, and seems to be essential for proper cellular localization[206]. The DH domain is the hallmark of Rho GEFs and constitutes the catalytic core of the Rho-GEF enzymatic activity for Rac and Cdc42. Several GEFs for small GTPases have been shown to bind both the GTPases and its downstream effector. Because there are more than 35 RhoGEFs and only a few Rho GTPases, the GEFs may be important for the selectivity of GTPase-effector interaction[196,207].

Both αPIX and βPIX show GEF activity for Rac and Cdc42, and can bind PAK proteins. The binding to PAK makes it possible that PIX transduces signals from Cdc42 to Rac[196,197,204](Figure 3.5). Furthermore, through this interaction, PIX co-localizes with PAK in focal adhesions and forms activated Cdc42- and Rac-driven focal complexes[196,208]. PIX proteins can therefore be placed in the previous described signaling pathways involved in regulation of the formation of lamellipodia and filopodia at neurite growth cones.

Molecular analysis of a reciprocal X/21 translocation in a male with mental retardation showed that the *ARHGEF6* gene in Xq26, encoding αPIX, is disrupted by the rearrangement. Mutation screening of 119 patients with nonspecific mental retardation revealed a mutation in MRX46 (*appendix 1 and 4*, this thesis). The intronic mutation in MRX46 leads to enhanced skipping of exon 2, and therefore to a partial deletion of the CH domain of the protein. The exon skipping in the affected males is not complete, meaning that there are some wildtype transcripts remaining that contain exon 2. Furthermore, unaffected individuals have up to 10% of exon 2 skipped product, indicating that a simple dominant negative effect of the mutation is unlikely. Probably, the MRX phenotype in family MRX46 is caused by a disturbance in the ratio between full-length and exon 2 (CH domain) skipped product. The mental retardation could be caused by a defect in the GEF activity of αPIX, and/or by a defect in the localization of the protein, because the CH domain is necessary for actin binding.

TM4SF2

Tetraspanins form a widely distributed protein superfamily[209,210], structurally characterized by four hydrophobic transmembrane regions (TM1-4) and two extracellular domains. The extracellular domains are known as large and small extracellular loops, with short intracellular amino and carboxy tails. The tetraspanin superfamily is composed of several subfamilies, and some 160 family members are known[211]. In humans, tetraspanins are localized in different organs and tissues. CD9, CD63, CD81 and CD82 are nearly ubiquitous, whereas other tetraspanins seem to be restricted to specific tissues, such as the lymphoid cells (CD53) or mature B cells (CD37). Tetraspanins are thought to be key players in the regulation of cell adhesion, proliferation, activation and migration[210]. Tetraspanins are known as molecular facilitators, associating in large cell-surface signaling complexes (referred to as the tetraspanin-web), which include integrins[212], co-receptors (CD4, CD8 and CD2)[213,214] and other tetraspanins[215,216]. Complex formation with ß-1 integrins is described by various authors[209,212]. Tetraspanins might recruit signaling

enzymes, such as PI 4-kinase[217], into complexes with integrins. It is hypothesized that these complexes should provide localized production of PtdIns-4-P, and subsequently PtdIns-4,5-P. These both can then be substrates for PI 3-kinase, and both are also established regulators of the actin cytoskeleton (Figure 3.6)[212].

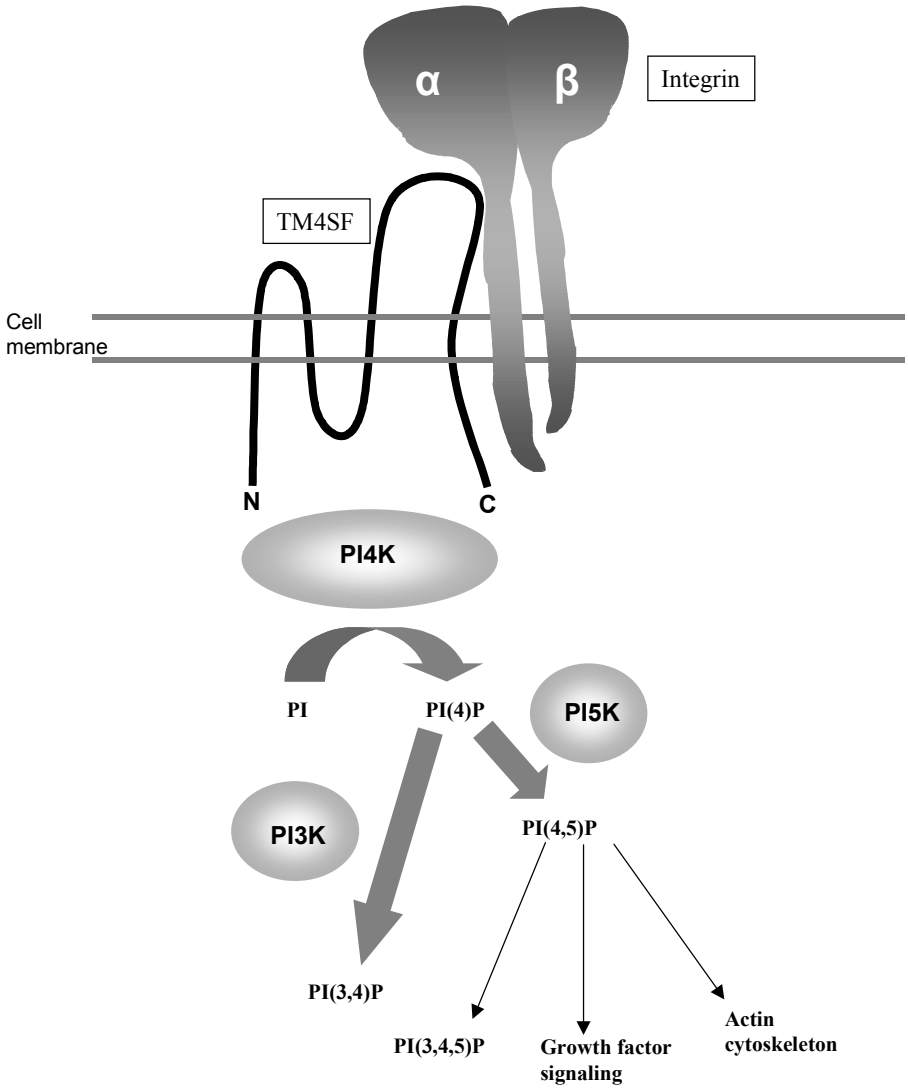

Figure 3.6. *Tetraspanins are involved in integrin signaling and play a role in neural adhesion. Cell adhesion may regulate the distribution of PI 4-kinase (PI4K), thus leading to a localized conversion of PI to PI(4)P, which is then converted by PI3K and PI5K to additional products (e.g. PI(3,4)P, PI(4,5)P and PI(3,4,5)P). PI(4,5)P then contributes to growth factor signaling and regulation of the cytoskeleton. (Adapted from Hemler[212])*

The *TM4SF2* gene at Xp11.4 codes for a tetraspanin protein[218], and is inactivated by the X-chromosomal breakpoint of a balanced translocation t(X;2)(p11.4;p21.3) in a female patient with MR and autistic behavior[49]. Subsequently, in two MRX families from the European MRX consortium mutations were found (missense and nonsense, respectively). TM4SF2 is ubiquitously expressed in fetal and adult tissues. The mouse Tm4sf2 is highly expressed in brain starting at a very early stage of differentiation (day E10.5). In adults, the highest levels of expression were observed in brain structures that correspond to the hippocampus and in the cortex, including the primary olfactory cortex[49]. The high level of

expression in structures known to be involved in memory development[152,219,220] suggests a role in physiological processes underlying memory and learning abilities.

The mutations found in *TM4SF2* gene are predicted to lead to defective integrin signaling. The disruption of the gene by the translocation leads to a barely detectable RNA transcript, whereas the nonsense mutation predicts a truncated protein lacking the fourth transmembrane segment and the carboxy-terminal domain. The missense mutation affects a non-conservative amino acid in the large extracellular loop[49,211]. Al three mutations therefore could lead to an abolished or reduced binding to β-integrin, and subsequently have an effect on neural adhesion and axon guidance[49].

IL1RAPL1

IL1RAPL1 and IL1RAPL2 are recently identified members of the interleukin-1 (IL-1) receptor protein family (IL1R), whose role in the IL-1 signaling mechanism was delineated in inflammatory response[221,222]. IL1RAPL1 has first been described by Carrié *et al*.[45] as IL1RAPL, and by Born *et al*.[223] as TIGIRR-2. IL1RAPL2 was known before as TIGIRR-1[223] and IL1-R9[224]. Both IL1RAPL proteins show homology to the known human IL1RAcP (IL1R accessory protein), which is encoded by the *IL1RAP* gene[221]. Like the other IL1R family members, IL1RAPL1 and -2 contain three predicted extracellular immunoglobulin (Ig) domains, a single transmembrane domain, and a highly conserved cytoplasmic region[223]. The C-terminal cytoplasmic region is extended relative to other IL1R family members, and is reminiscent of the Drosophila IL1R/Toll family members Toll and 18-Wheeler. However, sequence homology within the cytoplasmic domain clearly shows that these receptors are more similar to the IL1R family than to the Toll family[223].

The homology of the IL1RAPL proteins with IL1RAcP suggested a similar role in signaling events downstream of IL-1. IL1RAcP by itself has no measurable affinity for IL-1[225,226]. Upon binding of IL-1 to its receptor, however, a higher affinity binding complex is formed containing both IL1R and IL1RAcP[225]. IL1RAcP is also required for IL-1 signaling[227,228], via the IL1R-associated kinases (IRAK). IRAK in turn interacts with tumor necrosis-factor receptor-associated factor 6 (TRAF-6)[229,230], which plays a role in the activation of nuclear factor κB (NF-κB)[231]. IL-18 is structurally related to IL-1, and signaling by IL-18 is mediated by IL18R and IL18RAcP, both members of the IL1R family[232-234]. Neither IL1RAPL1 nor IL1RAPL2 could mediate NF-κB activation in response to IL-1 or IL-18[223,224]. It is possible that they bind IL-1 or IL-18, but activate an NF-κB-independent signaling pathway.

IL1RAPL1 is expressed at low level in fetal and adult brain[45,223]. In the mouse, *in situ* hybridization studies showed that it is first detectable in brain at day E10.5 and is upregulated at day E12.5, particularly in the hippocampus, parts of the cortex and the olfactory bulbs[45]. These brain structures are involved in the hippocampal memory system, suggesting therefore a specialized role for IL1RAPL1 in memory. IL-1 may regulate the process of learning and memory by modulating neuronal activity in the hippocampus[235].

Additionally, circulating IL-1 is able to act on hippocampal neurons to modulate the release of acetylcholine, a neurotransmitter involved in the regulation of memory processes[236,237]. IL1RAPL1 might therefore act downstream of IL-1 in the regulation of neuronal activity, via an up to now unknown signaling pathway.

Since the function of IL1RAPL1 is unclear, we can only speculate about the mechanism by which mutations in the *IL1RAPL1* gene cause MRX. Three of the eleven *IL1RAPL1* exons are deleted in MRX34, the entire gene is deleted in family P5 (MR-2), and in another family a nonsense mutation was detected in the last exon[45]. RT-PCR revealed that this mutation leads to a barely detectable *IL1RAPL1* transcript compared with that produced in normal controls. Both mutations therefore are predicted to be loss of function mutations.

The *IL1RAPL2* gene is also located on the X-chromosome (Xq22) and is expressed in fetal brain[223,224]. Therefore, it is a likely candidate gene for MRX. However, no mutations were found in the DNA from approximately 150 patients of the European MRX consortium (J Chelly, pers. comm.).

FMR2

Fragile sites appear as nonstaining regions, chromatid gaps, or, less frequently, as breaks in metaphase chromosome spreads. Fragile sites are highly conserved in evolution and can be found in many species, including primates, mouse, and horse, suggesting a common structure or function[238-240]. More than 100 fragile sites on human chromosomes have been reported to date, which are divided into two major groups. Fragile sites expressed in all individuals are called common fragile sites, the rest are called rare fragile sites. Both types are classified according to their induction conditions. Over 80 common fragile sites are known and induced by aphidicolin, 5-bromodeoxyuridine (BrdU) or 5-azacytidine. Twenty-five rare fragile sites in the human genome are induced in culture by the use of distamycin A, BrdU or folate depression. Five rare folate sensitive fragile sites have been identified at the molecular level (FRAXA, FRAXE, FRAXF, FRA16A, and FRA11B). These folate sensitive fragile sites are characterized by an expanded and methylated trinucleotide repeat of CGG or CCG[241-248]. Of the three X-chromosomal sites, FRAXA, FRAXE, and FRAXF, the former two are, in their expanded form, associated with mental retardation.

FRAXA expansion results in Fragile-X syndrome due to down regulation of expression of the *FMR1* gene in Xq28, which carries a polymorphic CGG repeat in the 5' untranslated portion of its first exon[249,250]. Normal individuals have between 2 and 50 copies of the CGG repeat. Repeat units larger than 50 CGGs become unstable and affected individuals have an amplification leading to more than 200 copies[241-243,251,252]. In individuals with Fragile-X syndrome, the CGG repeat and the adjacent CpG island are methylated[253,254], and the *FMR1* gene is transcriptionally inactive[249,250,255].

FRAXE is a rare fragile site in Xq28, approximately 600 kb distal to FRAXA[256,257]. An association of FRAXE expression and mild nonspecific mental retardation in males was first reported in 1993[248]. FRAXE expression involves the amplification of a (CCG)n repeat adjacent to a CpG island. Normal alleles vary from 4 to 39 copies[248,258,259]. In FRAXE expressing males more than 200 copies are found. Furthermore, the FRAXE associated CpG island in these males is fully methylated[248]. The incidence of FRAXE full mutations in the population has been estimated as 1 in 23,500[260]. In 1996, two groups cloned the first MRX gene, *FMR2*, which was located next to the fragile site. One of these groups studied two unrelated boys with submicroscopic deletions near FRAXE and mental retardation[47]. The other group used a large-scale sequencing approach[48].

The molecular basis of FRAXE has been identified as the absence or truncation[47,48] of the FMR2 protein. This is a consequence of either methylation of the FRAXE CpG island in individuals with the full mutation (> 200 CCG copies) and consequent transcriptional silencing of the *FMR2* gene[102], or deletion within the *FMR2* gene[47,102]. No *FMR2* specific point mutations have been identified so far. *FMR2* is expressed in adult brain and placenta, and in fetal brain, lung, and kidney[261]. Based on similarity with MLLT2/AF4[262], LAF4[263] and AF5q31[264], the FMR2 protein was predicted and subsequently demonstrated to localize to the cell nucleus[261]. Nuclear localization is consistent with its postulated function in transcriptional activation[261]. Little is known about the function of the AF4/FMR2 family of nuclear proteins. The recent identification of the *Drosophila* gene *lilliputian* (*lilli*) as a novel member of this family[265,266], provides the first insight into the function of this class of transcription factors. Lilli has a specific function in regulating the efficiency of signal transduction downstream of Raf, which is an effector of Ras. Raf activates the extracellular signal-regulated kinase (ERK)-mitogen activated protein kinase (MAPK) pathway, which will be discussed later in this chapter. As a putative transcription factor, Lilli may regulate the expression levels of one or multiple components of the Ras/MAPK signaling pathway[265]. Furthermore, Lilli regulates the expression of Sry α, a zygotic regulator of the actin cytoskeleton, and therefore is required for maintaining the actin network during cell growth and differentiation[266]. Based on its homology with Lilli, FMR2 might also be acting on the Ras/MAPK signaling pathway that is involved in maintenance of the actin cytoskeleton (Figure 3.7).

Some males with normal intellectual abilities and full methylation of the *FMR2* gene have been reported, either identified by a cytogenetically visible FRAXE expansion[248,256,267-269] or by the absence of an FMR2 transcript on Northern blot[102]. It has been speculated that the normal phenotype in these individuals is the result of the clinical variability of the disease, or mosaic methylation in different (especially neuronal) tissues[102,270]. Clinical variability may arise if the severity of the FRAXE MR phenotype is compensated by modifying factors of the genetic background or if the effect of FRAXE full mutation is so mild that the phenotype remains within the normal intellectual range in some individuals. The uncertain genotype-phenotype correlation in FRAXE-associated MR, however, might also be explained by the involvement of another gene than *FMR2*.

Recently, Gécz identified the *FMR3* gene, which is transcribed in the opposite orientation to *FMR2*[271]. The RNA transcript is most abundantly expressed in adult brain (medulla and spinal cord), but no ORF was present, indicating that there is no FMR3 protein formed. All FRAXE repeat expansions tested cause absence of both *FMR2* and *FMR3* transcripts[271]. On the other hand, the intragenic *FMR2* deletion does not affect *FMR3*, which makes the contribution of the *FMR3* gene to the FRAXE MR phenotype unlikely.

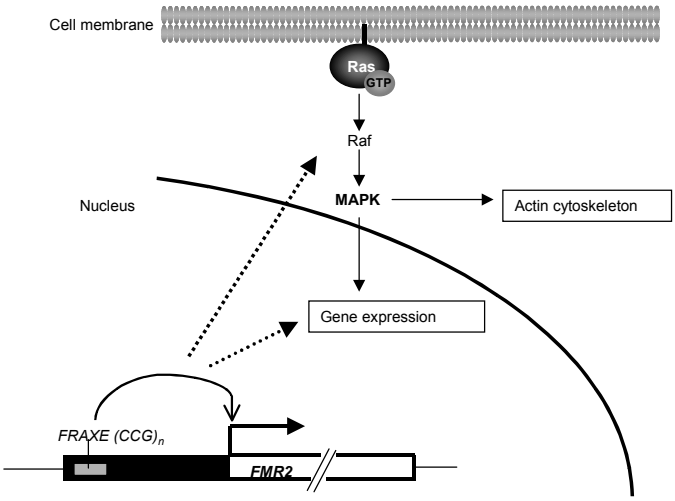

Figure 3.7. *Proposed function of FMR2. Dotted lines indicate likely connections in the intracellular pathway, based on homology with Drosophila gene lilliputian.*

RSK2

Mitogen-activated protein kinases (MAPK) are ubiquitous kinases involved in signal transduction in eukaryotic organisms[272-274]. The cascade is a three-kinase module comprising a MAPK, a MAPK kinase (MAPKK) and a MAPK kinase (MAPKKK)[274-276]. One of the best-studied MAPK cascades consists of the ERK MAPKs, the MAPK/ERK kinase (MEK) MAPKKs, and the Raf and Mos MAPKKKs. Raf and Mos can phosphorylate MEK on two serine residues, thereby activating them. Active MEK in turn phosphorylates ERK, making them active towards downstream substrates. Among the substrates of ERK are the family of p90 kDa ribosomal S6 kinases (RSK; also known as p90rsk or MAPK-activated protein kinase)[277-280]. RSK is unique among serine-threonine kinases in that it contains two functional kinase domains: an N-terminal domain that phosphorylates downstream substrates and a C-terminal kinase involved in the auto-activation of RSK. The RSK isoforms are activated by virtually all extracellular signaling molecules that stimulate the Ras-ERK pathway, i.e. growth factors and cytokines as well as many peptide hormones and neurotransmitters. ERK and RSK are present in the nucleus as well as in the cytoplasm[281].

The human RSK family of kinases includes four isoforms: RSK1, RSK2, RSK3[280,282], and RSK4 (*appendix 3*, this thesis). The isoforms are encoded by distinct genes and show 75-80% amino acid identity. The members of the RSK family show variable tissue expression, suggesting that they may be involved in different functions in the organism. RSK2 is the best studied family member. The substrates of RSK include transcription

factors like CREB, the estrogen receptor-α (ERα), NFκB and c-Fos[283-286] (Figure 3.8). Furthermore, RSK associates with the transcriptional coactivator proteins CREB-binding protein (CBP) and p300[287]. RSK binds to polyribosomes and phosphorylates several proteins in the ribosomal complex[288]. Finally, RSK has been shown to phosphorylate glycogen synthase kinase-3 (GSK3), the neural cell adhesion molecule L1CAM, the Ras GEF Sos, and the p34cdc2-inhibitory kinase Myt1[289-292]. The diversity of these substrates suggests that RSK is involved in regulation of a wide variety of cellular functions. Also histone H3 appears to be a direct or indirect target of RSK2, suggesting that chromatin remodeling might contribute to MAPK-regulated gene expression[293].

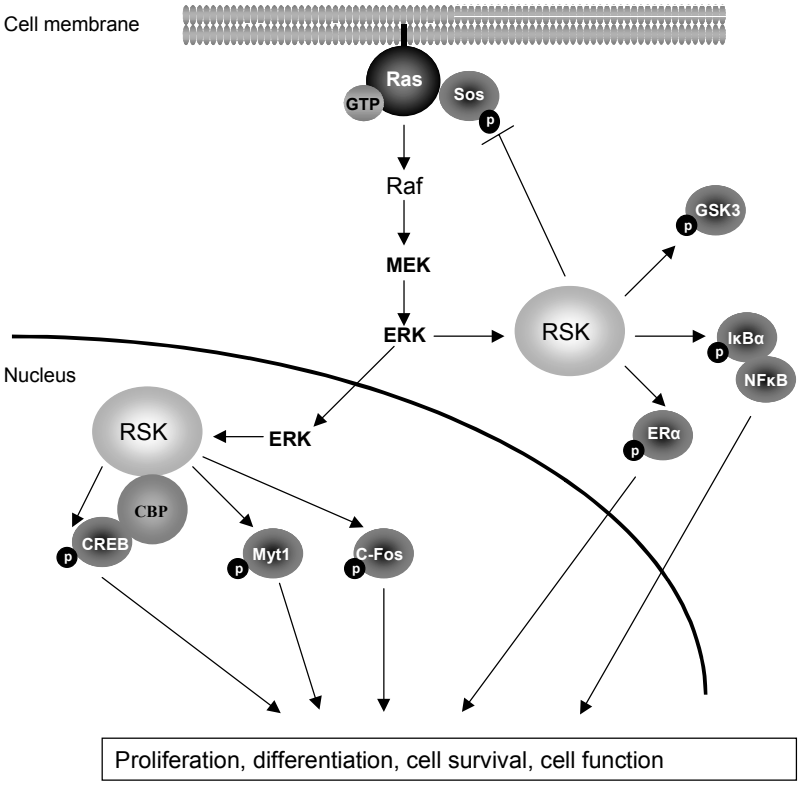

Figure 3.8. *Role of RSK in signal transduction. RSK is activated by ERK and mediates intracellular signals in response to growth factors, hormones and neurotransmitters that are capable of activating the Ras-ERK pathway. RSK phosphorylates cytosolic and nuclear proteins that are involved in diverse cellular processes, including gene transcription, protein synthesis and proliferation. (Adapted from Frödin and Gammeltoft[294])*

Coffin-Lowry syndrome (MIM 303600) is characterized by mental retardation, facial and digital dysmorphologies and progressive skeletal malformations. Analysis of the *RSK2* (*RPS6KA3*) gene in Xp22 in patients with this syndrome revealed intragenic deletions, nonsense and splice-site mutations that resulted in absent or truncated, nonfunctional proteins. Furthermore, several missense mutations were reported that also inactivate RSK2 kinase activity[295-297]. Interestingly, a missense mutation in a family with mild mental retardation without any dysmorphisms (MRX19) leads to 5-6 fold decrease in kinase activity[44]. Apparently, 15-25% of RSK2 activity is sufficient for normal skeletal development, but not for normal brain function. It cannot be excluded, however, that the missense mutation in MRX19 only affects the activity of RSK2 in brain cells. The involvement of RSK2 in brain function might be established via the phosphorylation of CREB, which has been implicated in LTP[298]. It is likely that the gene product of the *RSK4* (*RPS6KA6*) gene in Xq21 plays a similar role in brain development (*appendix 3*, this thesis), because deletions of this gene are involved in a contiguous gene syndrome, consisting of

deafness type 3 (DFN3), MRX and choroideremia (CHM). Until now there is no conclusive evidence that the *RSK4* gene is responsible for MRX, because no mutations were detected in almost 200 patients from the European MRX consortium. Furthermore, one patient with a partial deletion of the gene is not mentally retarded. However, the homology with *RSK2* still suggests a role in brain development, and functional studies and extended mutation screening may yet provide the definitive proof.

MECP2

The *MECP2* gene in Xq28 encodes methyl-CpG binding protein 2, a small protein that contains two recognizable domains: an 85 amino acid methyl-cytosine-binding domain (MBD), and a 104 amino acid transcriptional repression domain (TRD). The MBD binds to DNA that contains symmetrically methylated CpGs, whereas the TRD interacts with the transcriptional corepressor SIN3A to recruit histone deacetylases (HDACs). Interaction between this transcription repressor complex and chromatin-bound MECP2 causes deacetylation of core histones resulting in transcriptional repression[299-302]. Deacetylation of the core histones H3 and H4 alters the chromatin structure and renders the methylated DNA inaccessible to the transcriptional machinery. The MECP2 protein binds via its MBD to the promoter regions of certain genes that are enriched with CpG dinucleotides. The methylation of these CpG-islands regulates gene expression by altering chromatin structure, thereby "turning off" the gene. Rather than to suppress specific genes, MECP2 is thought to suppress the tissue-specific genes whose activity is not required in that particular cell type.

Mutations in the *MECP2* gene have been found in patients with Rett syndrome (RTT; MIM 312750), a progressive neurological developmental disorder and one of the most common causes of mental retardation in females, with an incidence of 1 in 10,000-15,000[303]. Patients with classic RTT develop normally until 6 to 18 months of age, then gradually lose speech and purposeful hand movements. They develop microcephaly, seizures, autism, ataxia, intermittent hyperventilation, and stereotypic hand movements. After initial regression, the condition stabilizes and patients usually survive into adulthood. Mutations in *MECP2* account for 70 to 80% of RTT cases and are involved in a broad spectrum of phenotypes[304-313]. The fact that such a globally applied mechanism as gene silencing results in the delayed onset and neuronal specificity observed in RTT suggests that specific genes are silenced by MECP2. These are presently unknown.

As RTT occurs almost exclusively in females, it has been proposed that RTT is caused by an X-linked dominant mutation with lethality in hemizygous males. Since the *MECP2* gene is subject to X-inactivation[314], random X-inactivation in the brain of heterozygous females might explain that mutations in females are not lethal. The presence of wildtype MECP2 in half of the cells is apparently sufficient for appropriate neuronal migration and correct formation and organization of brain structures. However, during the post-natal period of brain development, when synaptic connections are established and stabilized,

altered MECP2 activity and the resulting inappropriate expression of MECP2 target genes may have serious consequences for the development of cognitive function[315] (Figure 3.9).

Recent results indicate that *MECP2* mutations are not necessarily lethal in males. Mutations were first reported in severely retarded males with progressive neurological symptoms[316-318] and later in males with non-progressive encephalopathy[319], Angelman-syndrome phenotype[320], and even nonspecific XLMR[124]. Very recently, *MECP2* mutations in MRX16 and two other MRX families from the European MRX consortium have been reported[46,321]. Preliminary screening of sporadic unexplained MR, in which fragile-X screening was negative, indicates that *MECP2* mutations are found in 2% of cases[46]. When confirmed, *MECP2* has a contribution to MRX, equaling that of Fragile-X syndrome.

How *MECP2* mutations lead to mental retardation is unclear, since the downstream target genes are not yet known. Chromatin remodeling provides a mechanism by which the methylation state of a differentiated cell can be passed on through mitosis. It is possible that in neurons with defects in epigenetic gene silencing, normally inactive genes become active. It can be imagined that defects in gene regulation can lead to altered electrochemical activity in the nervous system and thus disrupt the establishment of synaptic connections[322]. Also, it is reasonable to expect that at least some genes regulated by MECP2 are critical for neuronal development, since small neuronal size and reduced dendritic arborization are commonly found in brains from RTT patients[323-325]. Furthermore, methylation has been implicated as a regulator of CREB function[326], indicating that DNA methylation might play a role in long-term learning and memory[327].

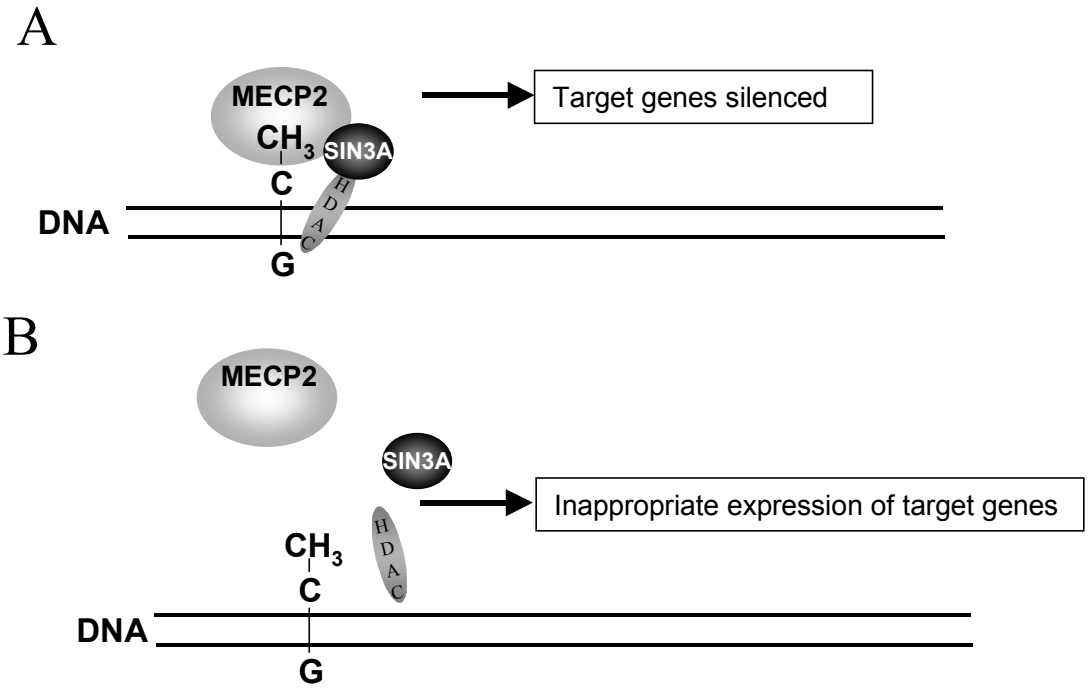

Figure 3.9. *MECP2 is mutated in RTT syndrome and MRX. (A) During normal development, MECP2 induces transcriptional repression of its target genes through binding of the MBD domain to 5-methyl cytosine on DNA (CH_3) and binding of the TRD domain to histone deacetylase (HDAC) and the transcriptional co-repressor SIN3A. (B) The transcriptional silencing complex is not formed with mutant MECP2, resulting in a mental retardation phenotype.*

X-inactivation plays an important role in the genotype-phenotype correlation of *MECP2* mutations. It is expected that favorably skewed X-inactivation in females may lead to the absence of clinical features. Indeed, such an X-inactivation pattern has been observed in healthy carriers of a *MECP2* mutation[305,316]. The phenotype of carrier females with random X-inactivation depends on the severity of the mutation. Significantly milder disease was noted in patients carrying missense mutations as compared with those carrying truncating mutations. And, in the case of truncating mutations, late mutations are associated with milder disease[308]. If the mutation is very mild, i.e. when it is not lethal in males, random X-inactivation in the carrier mothers might not even have a phenotypic effect. Indeed, random X-inactivation was reported in healthy carrier females[46,318,319]. Strikingly, carriers of the A140V mutation who showed random X-inactivation, were reported to have either no phenotype[46], or to have mild MR[124]. Since the X-inactivation pattern is studied in peripheral blood leukocytes, it is likely that non-random X-inactivation in neuronal cells is involved.

XNP

SNF/SWI proteins (homologues of S. cerevisiae "sucrose nonfermenter" and "switching defective") control transcription by opening the nucleosome structure at a promoter and by facilitating the binding of transcription factors to their cognate sites[328]. SWI/SNF can bind to either nucleosomes or DNA in an ATP-independent fashion[329,330], but the nucleosome remodeling activity of SNF/SWI is ATP-dependent[329,331]. SWI/SNF proteins form large, multi-subunit complexes, containing eight or more proteins. A "minimum catalytic core" complex of three SWI/SNF components, BRG1, INI1, and BAF155/BAF170, is necessary for nucleosome remodeling[332]. SWI/SNF complexes have been shown to contain either actin or actin-related proteins[333,334]. Actin might be required for SWI/SNF association with the nuclear matrix but not for its catalytic activity[334]. The finding that SWI/SNF also represses transcription[335,336] was unexpected, since the disruption of nucleosome structure would facilitate the binding of transcription factors, thereby activating transcription. However, repression of transcription by SWI/SNF could be caused by facilitated binding of transcriptional repressors, but also a mechanism independent of nucleosome remodeling activity could be involved, like histone modification[337]. It was suggested that, in higher eukaryotes, the epigenetic regulation of transcription by chromatin remodeling plays a critical role in the controlled expression of developmental programs and the stability of the cellular determined state[338-340].

The X-linked nuclear protein (XNP) belongs to the SNF2/SWI2 subfamily of helicase proteins[341,342]. The XNP protein contains a putative DNA-binding domain composed of three multicysteine zinc finger motifs, and a helicase domain composed of seven conserved motifs[343]. The helicase domain is expected to possess the ATPase activity and may be involved in chromatin remodeling by interacting with DNA using the energy of ATP hydrolysis[341,343]. The XNP protein participates in the formation of multiple protein

complexes and is able to associate with the human EZH2 protein[344], a human homologue of the *Drosophila* gene Enhancer of zeste, which is involved in the regulation of homeotic gene expression through chromatin remodeling[345]. Furthermore, XNP interacts with heterochromatin protein (HP1)[346]. XNP is a nuclear protein closely associated with the nuclear matrix at interphase and found close to pericentromeric chromatin during mitosis[347,348]. The XNP transcript is ubiquitously expressed and particularly abundant in human and mouse brain. In the developing mouse brain, the gene is highly expressed in areas where neural proliferation is occurring.

Mutations in the *XNP* gene in Xq13 have first been reported in ATR-X syndrome (MIM 309580), which is characterized by severe mental retardation, α-thalassemia, characteristic facial dysmorphisms, microcephaly and urogenital malformations[126]. Subsequent mutations have been found in a variety of other severe mental retardation conditions with facial dysmorphisms[127-131]. Recently, mutations have also been found in a family in which two affected males showed moderate to profound MR and had the typical characteristic features of ATR-X, but two others had mild MR and epilepsy and did not have the typical facial dysmorphisms[132]. This led to the hypothesis that the gene could be involved in familial cases of MRX and a mutation was found in a family previously diagnosed with MRX (*appendix 5*, this thesis). Retrospectively, some mild dysmorphic signs were seen in photographs taken in childhood, but the main conclusion that can be drawn from this finding is that males with an apparently nonspecific MR may carry a mutation in this gene.

The pathogenic effect of mutations in the *XNP* gene is unclear. Mutations in the zinc finger of the protein cause impaired nuclear location and altered DNA binding properties[349]. The exact mechanism of protein mislocalization is uncertain, and may be a consequence of failure of protein-protein interaction or a failure of DNA binding activity. All mutations associated with α-thalassemia are located within the helicase domain. It has been hypothesized that XNP regulates expression of several genes, including the α-globin gene, as well as other genes involved in development of the brain. Yet, target genes other than the α-globin gene have not been identified.

3.3 ROLE OF MRX GENES IN MEMORY DEVELOPMENT

Over the last three years, the repertoire of genes involved in MRX has expanded rapidly. Despite this high degree of apparent heterogeneity, two groups of MRX genes seem to exist: The first group (containing *MECP2* and *XNP*) plays a role in regulation of transcription via chromatin remodeling. The other group comprises those MRX genes that are directly involved in neuronal morphogenesis. Strikingly, most of the genes in the latter group can be placed in one signaling pathway that involves the Rho GTPases and MAPK. This pathway is directly involved in actin cytoskeleton organization that drives the outgrowth of neurons. *IL1RAPL1* and *GDI1* cannot directly be placed in either of these two

groups. IL1RAPL1 might be involved in the release of ACh in hippocampal neurons, thereby playing a role in the regulation of memory processes. GDI1 is involved in vesicle trafficking necessary for neuron outgrowth, and in the control of synaptic neurotransmitter release through Rab3A.

The known and proposed functions of all MRX genes are integrated in one model for memory development (Figure 3.11).

3.3.1 Repression of transcription

Selective expression of certain genes is necessary for the correct differentiation of a specialized cell type. It is not only essential to turn on the proper genes, but also to silence other genes. Inactive regions in the genome are characterized by condensed chromatin (heterochromatin), that is enriched with hypoacetylated forms of histones H3 and H4. The promoters of these genes are generally hypermethylated. Epigenetic silencing mechanisms like hypoacetylation and hypermethylation can be stably maintained through mitosis, and can therefore be passed on to all daughter cells.

DNA methylation in mammalian cells occurs at the 5-position of cytosine within the CpG dinucleotide, creating 5-methyl cytosine. There are about 45,000 CpG islands in the mammalian genome, most of which reside within or near the promoters or first exons of genes and are unmethylated in normal cells, with the exception of CpG islands on the inactive X-chromosome in females[350]. The unmethylated CpG islands have an open chromatin structure that is deficient in the linker histone H1 and contain nucleosomes enriched in acetylated forms of histones H3 and H4[351].

DNA methylation is established by DNA methyltransferases and leads to transcriptional silencing of the associated gene. Methyl-CpG binding proteins, including MECP2, selectively recognize methylated DNA and associate with co-repressor complexes that include histone deacetylases[301,302]. Recruitment of a histone deacetylase by MECP2 occurs indirectly through its interaction with the SIN3A adaptor protein, which causes transcriptional silencing, in part by deacetylating the histones[301,302]. This directs the formation of stable repressive chromatin structures[352] (Figure 3.10).

XNP is a member of the SNF2/SWI2 family of transcription factors. SNF/SWI proteins are able to alter the structure of nucleosomes via the formation of large, multi-subunit complexes. Although they were first thought to disrupt the nucleosome structure, therefore acting as transcriptional activators, it is now known that they remodel nucleosomes between two states: the "inactive" state and the "remodeled" state, with equal proficiency[353,354]. XNP is thought to regulate gene expression by altering chromatin structure via direct binding to heterochromatin-associated proteins. XNP interacts with heterochromatin protein (HP1)[346] and with EZH2[344], which is implicated in the assembly of repressive protein complexes and is involved in gene silencing. Apparently, also here the nucleosome remodeling leads to a facilitated binding of transcriptional repressors. It is unclear, however, how the interaction of XNP with EZH2, forming a transcription

repression complex, leads to the activation of α-globin expression. Interestingly, the human α-globin cluster was found to be located in a segment of chromatin which becomes hyperacetylated when the α-genes are fully active in erythroid cells[355]. Furthermore, patients with ATR-X syndrome also have methylation defects, which include both hypo- and hypermethylation at certain repetitive elements[356]. Since DNA methylation and histone deacetylation precede the chromosome remodeling, it is likely that XNP acts on one of these processes. It has been hypothesized that XNP might be a member of a HDAC/MECP complex involved in establishing or maintaining the pattern of methylation in the genome[356].

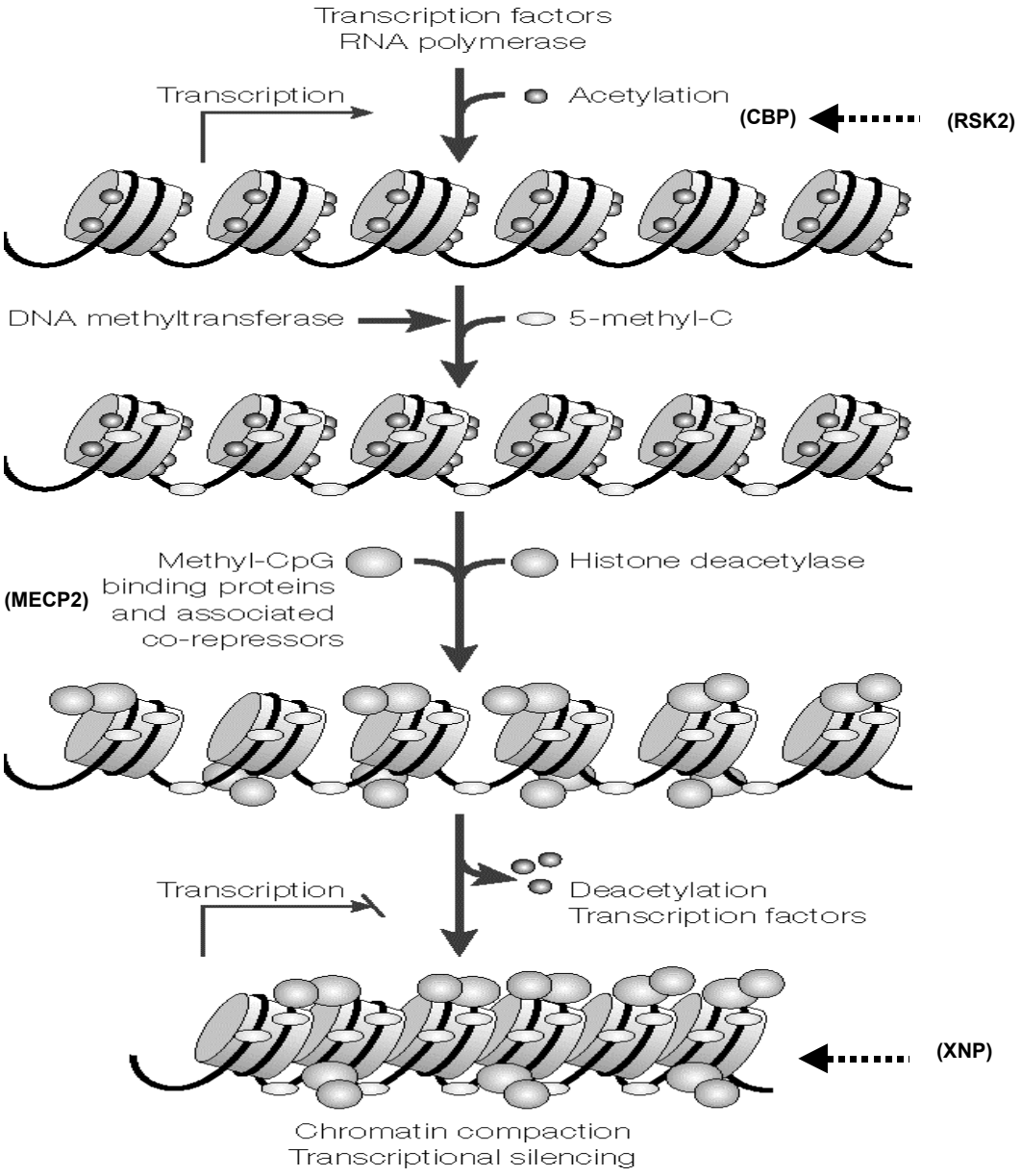

Figure 3.10. *The mechanism whereby DNA methylation and histone deacetylation cooperate to repress transcription. A transcriptionally active region targeted for silencing is proposed to acquire DNA methylation first, which then recruits the methyl-CpG binding proteins and their associated co-repressors and histone deacetylases. As DNA methyltransferase can interact directly with histone deacetylase, it is also possible that transcription is first silenced by deacetylation by other tethering factors, after which the methylation machinery and the methyl-CpG binding proteins are recruited to "cement" the promoter in the silent state. In either case, the deacetylated nucleosomes adopt a more tightly packed structure that inhibits the access of transcription factors to their binding sites. (Adapted from Robertson and Wolffe[357])*

Mutations in *MECP2* and *XNP* both lead to a variety of mental retardation phenotypes, depending on the severity of the mutation. Both genes are highly expressed in brain. Chromatin remodeling and methylation of DNA apparently are essential for normal brain development. DNA methyltransferase activity is high in neurons[358], indicating that gene silencing is very important for the correct function of neurons (during brain development and in adult life), allowing only the essential genes to be expressed. Activation of only a few genes that are normally inactivated during neuronal morphogenesis can have dramatic consequences for the correct functioning of learning and memory processes. The elucidation of which genes are normally silenced by MECP2 and XNP will give more insight in the pathogenesis of mental retardation.

Finally, also the RSK2 protein plays a role in chromatin remodeling, via the phosphorylation of histone H3 upon stimulation with epidermal growth factor (EGF)[293]. The growth-factor dependent interaction of RSK2 with the transcriptional coactivator CREB-binding protein (CBP)[287], a histone acetyltransferase[359], raises the possibility that histone acetylation and phosphorylation may act together to facilitate gene expression[293].

3.3.2 Signaling through Rho GTPases and MAP kinases

As described in the first paragraph of this chapter, neuronal development starts with the outgrowth of axons. The formation of filopodia and lamellipodia is regulated by the Rho GTPase cascade. Activation of Cdc42 leads to activation of Rac and finally to activation of Rho. MRX genes that can be placed directly in this cascade are *ARHGEF6*, *OPHN1*, and *PAK3*. OPHN1 acts as a GAP on Cdc42, Rac and Rho. Mutations in this gene therefore lead to an abolished GAP activity, meaning that the switch from the GTP-bound to the GDP-bound form of the Rho GTPases cannot take place, leaving them constitutively active. αPIX, the product of the *ARHGEF6* gene, acts as a GEF on Cdc42 and Rac, but also binds to PAK. PAK3 is a downstream effector of Cdc42 and Rac. Mutations in both genes therefore have an effect on the normal Cdc42-Rac-Rho pathway that is necessary for the formation of filopodia and lamellipodia, via modulation of actin cytoskeleton organization in the growth cone.

Axon guidance is dependent on neural adhesion via binding to molecules like integrins. The activation of the MAPK pathway by Rho GTPases appears to be integrin dependent[360]. It is possible that TM4SF2, through its interaction with β-1 integrins, plays a role in activation of the MAPK pathway, although the mechanism is not yet elucidated. PAK can directly phosphorylate Raf, a MAPKKK, which in turn activates MEK (MAPKK) and ERK (MAPK). ERK can activate the RSK2 protein, both in the cytoplasm and in the nucleus. The subsequent phosphorylation of downstream substrates of RSK2 ultimately leads to the maintenance of the actin cytoskeleton and formation of memory via the activation of CREB. Although not proven, the FMR2 protein might play a similar role, based on its homology with the Drosophila lilliputian that regulates the MAPK pathway and is also thought to be involved in the maintenance of the actin cytoskeleton[265,266].

Combining the proposed functions of the MRX genes described above into a model (Figure 3.11) gives insight in the pathways involved in the development of memory processes. Loss of function mutations in the MRX genes involved in this pathway suggest that the mental retardation could be caused by impaired ability of actin cytoskeleton organization to drive neurite outgrowth leading therefore to aberrant neuronal morphogenesis, dendritogenesis and connectivity between neuronal cells. Obviously, other genes involved in this pathway are candidate genes for mental retardation.

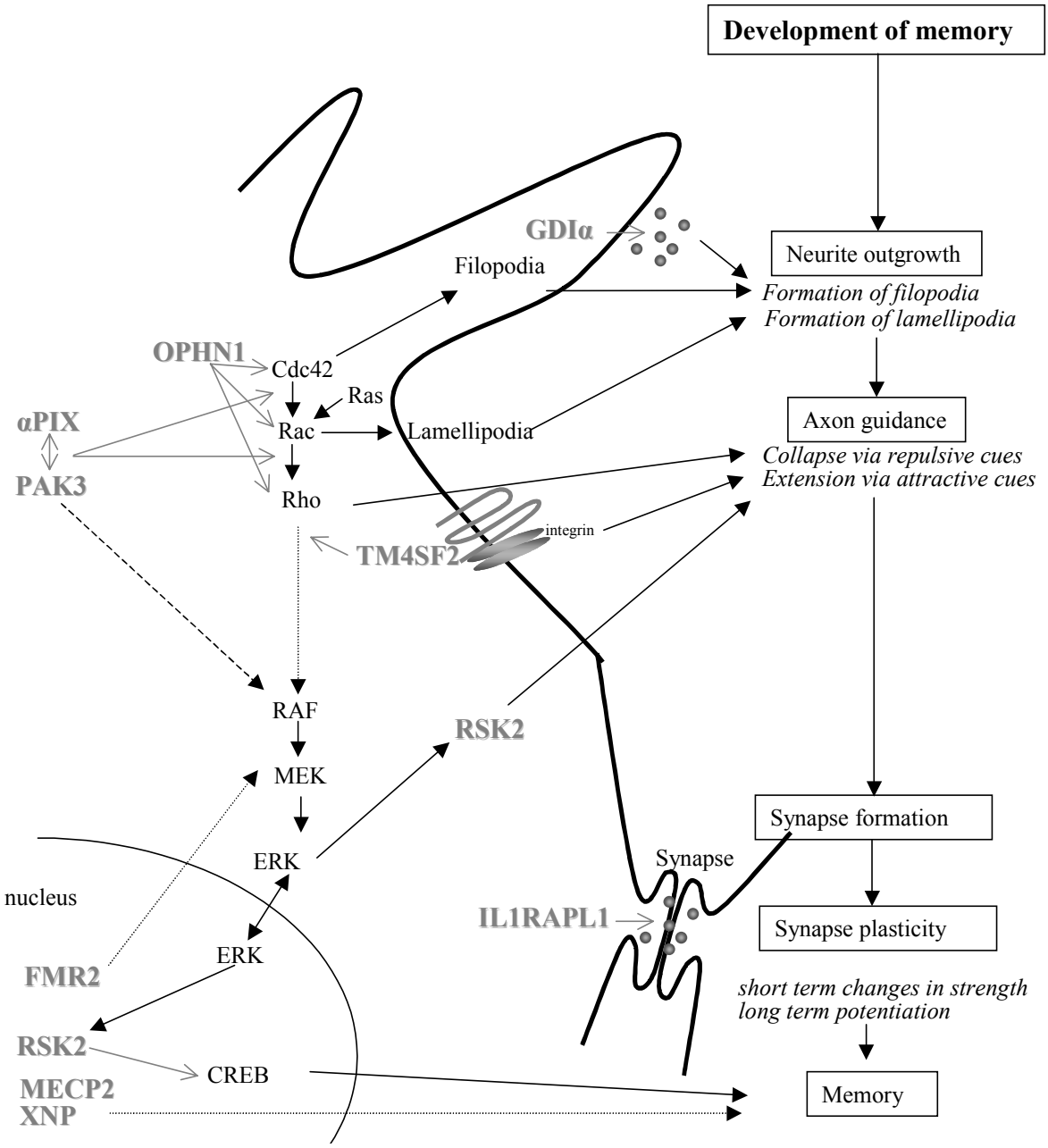

Figure 3.11. *Schematic involvement of the currently known MRX genes (depicted in gray). Dotted lines indicate likely connections in the pathway.*

Chapter 4

Conclusion and future prospects

4.1 MRX GENES

As one could perhaps have predicted from the complexity of the CNS, the MRX genes encode a range of proteins, including transmembrane proteins, transcription factors, kinases and proteins that regulate members of the Ras superfamily of small GTP-binding proteins. Up to now, molecular studies performed by 1) FISH analysis and physical mapping of X-chromosome rearrangements in mentally retarded individuals (positional cloning), and 2) by testing candidate genes that map in the X-chromosome regions defined by linkage studies (positional candidate gene approach), have identified seven genes that are specifically mutated in MRX families: *FMR2*[47,48], *GDI1*[39,40], *OPHN1*[41], *PAK3*[42,43], *IL1RAPL1*[45], *TM4SF2*[49], and *ARHGEF6* (*appendices 1 and 4*, this thesis). Furthermore, in two MRXS genes mutations have been found in families with MRX: *RSK2*[44], and *MECP2*[46]. Now that essentially all human genes have been identified by the human genome projects, the strategies for detecting novel MRX genes need to be reassessed. Specifically, the candidate gene approach will be most effective in future identification of novel MRX genes. Positional cloning approaches do not always lead to the identification of novel genes. Furthermore, genes that are already known to be involved in mental retardation syndromes, now are excellent candidate genes for nonspecific MR. A good example is the *XNP* gene. The MRX family in which a mutation was found (*appendix 5*, this thesis) showed minimal characteristics of ATR-X syndrome, but only on retrospective analysis. These results indicate that mild syndromic features in childhood can disappear in adulthood. MRXS genes involved in a broad clinical spectrum should be regarded as candidate genes for MRX.

Two main processes seem to be affected by mutations in the MRX genes. The first is the global gene silencing in neurons during development. For the developing neuron it is essential that the correct genes are turned on. It is of equal importance that other genes are turned off. Both MECP2 and XNP play a role in this global gene silencing process. The other process involved in MRX is the intracellular pathway that is initiated by Rho GTPases. These are involved in actin cytoskeleton rearrangement, that is necessary for the correct outgrowth of filopodia and lamellipodia from the growth cone of a neuron. Direct regulation of these Rho GTPases takes place via OPHN1, αPIX, and PAK3. Downstream of this pathway the MAPK pathway is activated, probably via interference of TM4SF2 and FMR2. RSK2 has a function downstream of the MAPK pathway. RSK2 can phosphorylate CREB in the nucleus. This protein in turn is essential for LTP and memory.

All MRX genes are expressed early in development, and most of them are expressed in the hippocampus in the adult brain. This brain structure is considered one of the important regions for learning and memory, and alterations, both during development and postnatally, may account for mental handicap. The study of mouse knock-outs for the MRX genes, which are under way in different laboratories, will help to clarify some of the underlying pathogenic mechanisms.

4.2 THE X-CHROMOSOME AND INTELLIGENCE

4.2.1 How many MRX genes exist?

In 1996, delineation of the number of non-overlapping MRX localizations led to a minimum estimate of eight genes[51]. This estimate has since been increased to 17, by including the seven identified MRX genes and two genes for syndromic and nonspecific XLMR. In the most recent compilation eight non-overlapping regions can still be counted (see Figure 2.1, this thesis). As only in 9 of 76 MRX families (Table 4.1) a mutation has been identified, only 12% of cases has been solved. On average, each MRX gene identified until now, is mutated in approximately 1% of patients tested. The overall number of X-linked genes implicated in mental retardation may therefore be substantially more than 17. A better estimation can be made if also the mutations found in small (unlinked) families are counted. The European MRX consortium has searched for mutations in seven of the MRX genes (*GDI1*, *OPHN1*, *PAK3*, *IL1RAPL1*, *TM4SF2*, *ARHGEF6*, and *MECP2*) in approximately 150 patients. In these seven genes in total 16 mutations were found (Table 4.1), which is still only 10% of tested patients. The *MECP2* gene was found to be mutated in four families, meaning that this gene alone contributes to more than 2% of MRX cases. Analyses in a large cohort of mentally retarded males, for whom the fragile-X diagnostic test was negative, indicates also *MECP2* mutations in 2% of cases[46].

Table 4.1. *Genes involved in MRX*

Gene	Means of identification	Mutation in MRX family	Mutation in small family
FMR2	Positional cloning [47]	-	-
GDI1	Functional candidate	MRX41, MRX48[40]	Family R (P6)[39]
OPHN1	Positional cloning	MRX60[41]	-
PAK3	Functional candidate	MRX30[42], MRX47[43]	-
RSK2	MRXS gene[295]	MRX19[44]	-
IL1RAPL1	Positional cloning	MRX34[45]	N36, P5[45]
TM4SF2	Positional cloning	-	L28, T15[49]
ARHGEF6	Positional cloning	MRX46 (*appendices 1 and 4*, this thesis)	-
MECP2	MRXS gene[304]	MRX16[46]	L26[318], T36[46], T44[321]
XNP	MRXS gene[126]	-	N29 (*Appendix 5*, this thesis)*

*This family was implicated in the European consortium panel of 200 MRX patients, but after the XNP mutation was identified, childhood hypotonia and HbH inclusion bodies were found in some patients of the family.

One could argue that not in all 150 patients of the European XLMR consortium an X-chromosomal gene is involved, and that the actual number of MRX patients tested is lower. This cannot be excluded. The European XLMR consortium includes only families with more than two affected males, and in which the disease is only transmitted by females. Even in the families with the lowest criteria, i.e. two affected brothers, it has been estimated that there is 80% chance that the mental retardation is the result of a defect in an X-linked gene[361]. Thus, it is likely that X-linked genes are involved in most of the families from the European XLMR consortium.

Some X-linked genes involved in chromosomal aberrations associated with MR have been described, in which up to now no mutation in MRX families has been found. At least

four of these genes exist: *DXS6673E*[106], *GRIA3*[107], *VCX-A*[108], and *RSK4* (*appendix 3*, this thesis). The *RSK4* gene is particularly interesting, because of its high homology with *RSK2*. Future mutation analysis of three novel exons may yet reveal a mutation in MRX. Although other genes might be located in the MRX critical interval in Xq21, this is unlikely, given the paucity of ESTs and predicted genes in the region (http://genome.cse.ucsc.edu/).

Taking all into account, it seems fair to expect 50 or more MRX genes on the X-chromosome. Although unlikely, it is still possible that there is a small number of major genes which are frequently mutated and account for a significant proportion of mentally retarded patients. Some authors have reported that the distribution of the genetic loci over the X-chromosome is not "at random", and that there is a clustering of MRX loci around the centromere. Indeed, looking at the overlap of linkage intervals in the 76 MRX families reported, one would expect a hotspot for mutations in the pericentromeric region of the X-chromosome, with the highest mutation frequency in Xp11.23 (Figure 4.1). However, the

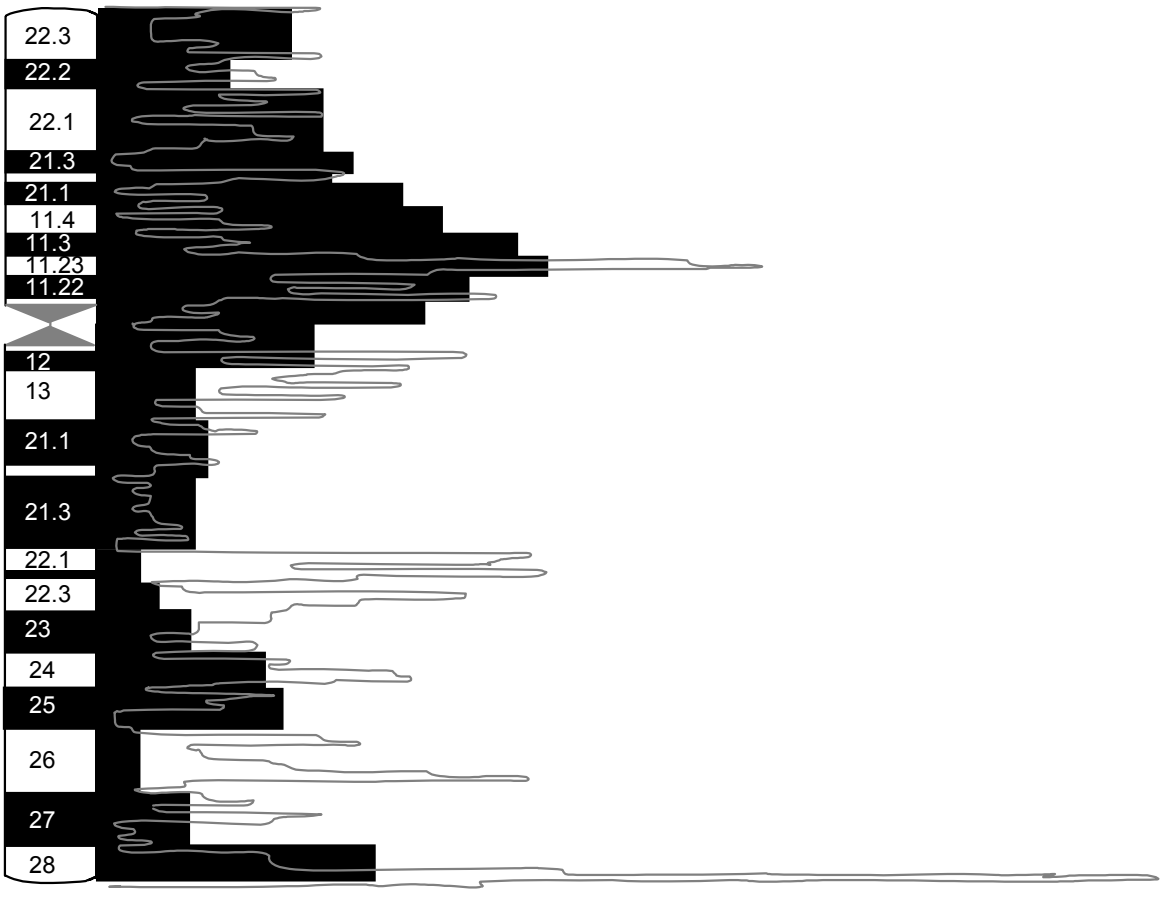

Figure 4.1. *Distribution of linkage data in MRX family and genes on the X-chromosome. Black bars indicate the distribution of MRX linkage intervals, normalized for the size of the intervals. Shortly, linkage intervals were given a fixed value of 100, divided by the size of the linkage interval in megabases. This means that the probability of the presence of an MRX gene in a certain chromosome band is higher if the linkage interval is small. Per chromosome band, the calculated values of the MRX families linked to the band were added. The gray line indicates the gene density on the X-chromosome. (Adapted from combined sequence data of the Sanger and EMBL centers, at http://www.ensembl.org/perl/mapview?chr=X)*

high number of families linked to this region does not necessarily mean that this region contains an unknown MRX gene that is frequently mutated. In the same figure the gene density on the X-chromosome is depicted, and Xp11.23 appears to contain more genes than other regions. Probably more MRX genes are located in this region. The most prominent difference between gene density and the MRX linkage data is seen in Xq28. However, already three MRX genes are identified in this band: *FMR2*, *GDI1* (mutated in MRX41 and MRX46), and *MECP2* (mutated in MRX16).

MRX gene identification may be "easiest" in regions with low gene density, but where a lot of MRX families are linked. In this view, Xp21.3 and the region from Xp21.1 to Xp11.3 are good candidate regions.

4.2.2 Are there any autosomal MR genes?

Several authors have posited the idea that a concentration of genes on the X-chromosome concerned with intelligence may be a major explanation of the male excess in MR[9,14,362-366]. Indeed, a number of X-chromosomal genes for nonspecific MR have been identified, whereas no autosomal genes are known. Further evidence for the importance of X-linked genes that influence intelligence is provided by the fact that there is greater variability of intelligence among males[367]. Males have fewer performers in the middle (mean) range than females, and more in the extremes beyond 2 standard deviations. It has been hypothesized that because of hemizygosity for genes on the X-chromosome, males feel the full influence of these intelligence genes, whether positive or negative. In females, a second X-chromosome serves to modify the influence, lessening the impact of positive or negative influences of specific genes for intelligence[9]. One would expect that on the X-chromosome also genes for superior intelligence exist. No variations in X-linked genes have been identified that might account for higher IQ (>130), but such families have not been sought or investigated.

There is a strong bias toward identifying genes on the X-chromosome. Against the idea of the disproportionate influence of the X-chromosome in determining intelligence is the observation that microdeletions and subtelomeric deletions almost anywhere in the autosomal genome produce mental retardation[17,368]. Given the broad spectrum of genes that are expressed in the brain during and/or following development, it is expected that numerous autosomal genes are involved in cognitive function. Several autosomal genes have been identified for syndromes in which mental retardation is a recurrent feature, but no autosomal genes for nonspecific mental retardation have been described. Current research into the identification of autosomal genes is focused on screening patients with idiopathic MR for submicroscopic rearrangements and deletions particularly in telomeric regions. Several such rearrangements have recently been identified[17,109,368,369]. Knight *et al*.[368] described subtle chromosomal abnormalities in 7.4% of children with unexplained moderate to severe MR, indicating that subtle chromosomal rearrangements are the second most common cause of moderate to severe MR after Down's syndrome.

Homozygosity mapping in consanguineous families with affected offspring would be another approach[370]. Linkage analysis revealed only one autosomal locus for nonspecific mental retardation (without additional phenotypic characteristics)[371]. Autosomal recessive mental retardation is probably not always recognized and is likely to be highly genetically heterogeneous. There are not many autosomal dominant pedigrees with MR expected, because except for milder forms, these individuals do not reproduce. Identification of autosomal genes involved in cognitive function will benefit from knowledge accumulating in the field of XLMR, as these genes will likely encode proteins that play a role in the same molecular pathways. If the number of MRX genes does exceed 50 (but even if there are less) and if genes essential for normal cognitive function are randomly distributed in the genome, the level of complexity of the underlying molecular basis for mental retardation will be enormous[370].

4.3 Future prospects

To date, causative genetic defects were identified for 17 families with nonspecific X-linked mental retardation. Since it is expected that there might be more than 50 MRX genes, the identification of novel genes remains a challenge. Positional cloning has so far proven the most efficient way to identify MRX genes. In some cases, however, the breakpoint-spanning fragments have been cloned and sequenced, but no gene could be identified. Also, positional cloning is a time-consuming method, and the involvement of the cloned gene in MRX can be proven only when a mutation in another family is found. Identification of MRX genes will thus require systematic testing of many candidate genes for mutations in probands of families with compatible localization. One could envisage to directly screen candidate genes on such a cohort of patients. Indeed, in the *TM4SF2* gene, mutations were found in two small families without significant linkage data. The identification of novel MRX genes therefore requires the analysis of a large number of MRX families, both large linked families and families that are too small to obtain a significant lod score.

The availability of probands may thus constitute a limiting factor in the identification of novel MRX genes. In order to overcome this limitation it will be necessary to join forces and to exchange patient material. Establishment of the European XLMR consortium was the first initiative to construct a large patient panel, which has resulted in a collection of approximately 200 well-characterized XLMR families. Most of these families have nonspecific MR. Inclusion criteria are the existence of at least two affected males in the family, and only females transmitting the disease. In some pedigrees, carrier females might show milder mental retardation.

Given the necessity to test large numbers of patients, high-throughput mutation analysis is required for identification of novel MRX genes. Nowadays direct sequencing on a 96 capillary machine, with or without pre-screening protocols like SSCP, DGGE or D-

HPLC, is the fastest way to perform such analyses. Once the conditions for analysis of mutations are defined for a given gene, they can be used easily on a relatively large number of samples in a single experiment. For individual molecular genetics laboratories it is time consuming and too expensive to test all 1500 X-chromosomal genes in 200 patients. Only if direct sequencing reactions become less expensive and/or collaboration with large genome sequencing centers provide a greater capacity for mutation analysis, such a large project can be successful. Until then, it will be useful to select candidate genes on positional and/or functional information. Positional information can be obtained by traditional positional cloning approaches. In the near future the identification of microdeletions on microarrays will also provide information on the position of MRX genes. Genes that act on transcriptional regulation or play a role in signaling pathways through Rho GTPases and MAP kinases are excellent functional candidate genes for MRX. Furthermore, additional MRX candidate genes might be identified by their altered expression in MRX patients with chromosomal aberrations[372]. In this way genes can be identified that are either directly involved in the chromosomal rearrangement, or indirectly via a position effect.

Therapy for nonspecific X-linked mental retardation will not be possible in the near future. Future studies have first to give more insight in the mechanisms leading to MRX, and in the microstructure of the brains of MRX patients. For neurodegenerative disorders, a possible new therapeutic neurological and neurosurgical methodology involves cell implantation into the living brain in order to replace neuronal systems. Embryonic stem cells implanted in the brains of mice and rats develop into neurons and make renewed connections[373], and even adult cells from the bone marrow can enter the brain in mice and become neuron-like cells[374]. Pilot studies in humans with Parkinson's disease gave promising results, although the optimal cells for replacement cannot be reliably obtained or generated in sufficient numbers for a standardized medically effective intervention[375]. In any case, neurodegeneration (progressive neuron loss) and neurodevelopment are different mechanisms in the brain. Therefore, such therapy protocols might not even be applicable to treat mental retardation. Of equal importance, the use of gene- and cell based therapies, and whether or not this research should be pursued on mentally retarded individuals is also subject of ethical debates.

For the families in which the genetic defect is found, genetic counseling becomes possible. For some families linkage data alone may be beneficial, as a means of carrier identification, prenatal detection, and postnatal diagnosis. With few exceptions, the only DNA diagnostic test is offered for the Fragile-X syndrome. *FMR1* repeat expansion explains approximately 2% of all MR in males[376]. If it turns out that the *MECP2* mutations are equally frequent, diagnostic screening for mutations in this gene should be implemented. The other MRX genes contribute only 0.5-1% to the prevalence of MRX. It will be hard to develop a cost-effective screening for these genes in a DNA-diagnostic laboratory. With today's DNA-diagnostic protocols it is feasible to test only these genes that are more frequently causally involved in MR. To increase the number of positive

DNA-based diagnoses in MRX it will be necessary to include several genes in one test. A good test will give the most positive results with the lowest number of exons tested. Therefore, an inventory has to be made of the contribution of each gene to MRX. Those genes that are most frequently mutated in a panel of well-defined MRX families (e.g. those from the European XLMR consortium) have to be tested in an even larger panel of patients for whom the Fragile X screening was negative. The genes in which the most mutations will be identified in the latter group are candidates to include in a diagnostic test.

The ultimate scientific goal is not just finding the genes for MRX, but understanding how they function. Knowledge of the complete set of MRX genes will undoubtedly suggest new areas of investigation of neuronal function. Insight in the organization and functioning of the human brain can be obtained by cognitive neuroimaging, both in healthy and in mentally retarded individuals. Such in vivo measurements can produce pictures that shed new light on brain functions ranging from the processing of sensory information to higher level thinking tasks. A major approach in cognitive neuroimaging is to scan the brain's hemodynamic activity during the execution of a cognitive task. Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) may in the future focus on those brain areas in which MRX genes are known to be specifically active. Based on knowledge of the underlying genetic pathophysiological mechanism, future functional imaging as well as neuropsychological testing (and combinations of these) could also be fine-tuned to specifically target biological and physiological processes that are predicted to be affected. In this way, the current rapid increase of the basic genetic level may soon translate into a better functional understanding of human brain function. It will take many years before all of this is of practical benefit to the mentally retarded patient. For now it is encouraging that at least some progress has been made in understanding the molecular mechanisms underlying human brain function.

References

1. American Association on Mental Retardation (1992) Mental Retardation: Definition, classification and systems of support, 9th edition. Washington DC: American Association on Mental Retardation.
2. Stevenson RE, Schwartz CE, and Schroer RJ (2000) X-linked mental retardation. New York, Oxford: Oxford University Press.
3. Birch HG, Richardson SA, Baird D, Horobin G, and Ilsley R (1970) Mental subnormality in the community: a clinical an epidemiological study. Baltimore MD: Williams and Wilkins.
4. Laxova R, Ridler MAC, and Bowen-Bravery M (1977) An etiological survey of the severely retarded Hertfordshire children who were born between January 1, 1965 and December 31, 1967. *Am J Med Genet* **1**: 75-86.
5. McLaren J, and Bryson SE (1987) Review of recent epidemiological studies of mental retardation: prevalence, associated disorders, and etiology. *Am J Ment Retard* **92**: 243-254.
6. Hagberg B, and Kyllerman M (1983) Epidemiology of mental retardation - a Swedish survey. *Brain Dev* **5**: 441-449.
7. Penrose LS (1938) A clinical and genetic study on 1280 cases of mental defect. Special report series, Medical Research Council, vol 229. London: Her Majesty's Stationary Office
8. Hamel BCJ (1999) X-linked mental retardation; a clinical and molecular study. Alkmaar NL: Dékavé. pp 9-28.
9. Lehrke RG (1974) X-linked mental retardation and verbal disability. *Birth Defects Orig Artic Ser* **10**: 1-100.
10. Herbst DS, and Miller JR (1980) Nonspecific X-linked mental retardation II: the frequency in British Columbia. *Am J Med Genet* **7**: 461-469.
11. DeBow JDB (1854) Compendium of the seventh census:1850. pp 60,78.
12. U.S. Department of the Interior (1883) Census of United States 1860 (eight), 1870 (ninth) and 1880 (tenth). Washington: Government Printing Office.
13. U.S. Department of the Interior (1894) Compendium of the Eleventh Census: 1890. part II. Washington DC: Government Printing Office. pp 133-134.
14. Turner G (1996) Finding genes on the X chromosome by which *Homo* may have become *sapiens*. *Am J Hum Genet* **58**: 1109-1110.
15. Turner G, and Turner B (1974) X-linked mental retardation. *J Med Genet* **11**: 109-113.
16. Hamel BCJ, and Smeets DFCM (1997) Mentale retardatie. *Tijdschr Kindergeneeskd* **65**: 224-227.
17. Flint J, Wilkie AO, Buckle VJ, Winter RM, Holland AJ, and McDermid HE (1995) The detection of subtelomeric chromosomal rearrangements in idiopathic mental retardation. *Nat Genet* **9**: 132-140.
18. Knight SJ, and Flint J (2000) Screening chromosome ends for learning disability. *BMJ* **321** : 1240.
19. Moser HW, and Wolf PA (1971) The nosology of mental retardation: including the report of a survey of 1378 mentally retarded individuals at the Walter E. Fernald State School. *Birth Defects Orig Artic Ser* **7**: 117-134.
20. Fryns JP, Kleczkowska A, Dereymaeker A, Hoefnagels M, Heremans G, Marien J, and van den Berghe H (1986) A genetic-diagnostic survey in an institutionalized population of 173 severely mentally retarded patients. *Clin Genet* **30**: 315-323.
21. Hunter AGW, Evans JA, Thompson DR, and Ramsay S (1980) A study of institutionalized mentally retarded patients in Manitoba. I: Classification and preventability. *Dev Med Child Neurol* **22**: 145-162.
22. Gustavson KH, Hagberg B, Hagberg G, and Sars K (1977) Severe mental retardation in a Swedish county. I. Epidemiology, gestational age, birth weight and associated CNS handicaps in children born 1959--70. *Acta Paediatr Scand* **66**: 373-379.
23. Farag TI, Al-Awadi SA, El-Badramary MH, Aref MA, Kasrawi B, Krishna Murthy DS, El-Khalifa MY, Yadav G, Marafie MJ, Bastaki L et al (1993) Disease profile of 400 institutionalized mentally retarded patients in Kuwait. *Clin Genet* **44**: 329-334.
24. Anderson G, Schroer RJ, and Stevenson RE (1996) Mental retardation in South caroline II. Causation. *Proc Greenwood Genet Center* **15**: 32.
25. Kerr B, Turner G, Mulley J, Gedeon A, and Partington M (1991) Non-specific X-linked mental retardation. *J Med Genet* **28**: 378-382.
26. Chiurazzi P, Hamel BCJ, and Neri G (2001) XLMR genes: update 2000. *Eur J Hum Genet* **9** : 71-81.
27. Suthers GK, Turner G, and Mulley JC (1988) A non-syndromal form of X-linked mental retardation (XLMR) is linked to DXS14. *Am J Med Genet* **30**: 485-491.
28. Mulley JC, Kerr B, Stevenson R, and Lubs H (1992) Nomenclature guidelines for X-linked mental retardation. *Am J Med Genet* **43**: 383-391.
29. Opitz JM, Segal AT et al (1965) X-linked mental retardation:study of a large kindred with 20 affected members. *J Pediatr* **67**: 713.
30. Herbst DS (1980) Nonspecific X-linked mental retardation I: a review with information from 24 new families. *Am J Med Genet* **7**: 443-460.
31. Herbst DS, Dunn HG, Dill FJ, Kalousek DK, and Krywaniuk LW (1981) Further delineation of X-linked mental retardation. *Hum Genet* **58**: 366-372.
32. Tariverdian G, and Weck B (1982) Nonspecific X-linked mental retardation - a review. *Hum Genet* **62**: 95-109.
33. Gedeon A, Kerr B, Mulley J, and Turner G (1994) Pericentromeric genes for non-specific X-linked mental retardation (MRX). *Am J Med Genet* **51**: 553-564.
34. Yarbrough KM, Howard-Peebles PN (1976) X-linked nonspecific mental retardation. Report of a large kindred. *Clin Genet* **9**: 125-130.
35. Wilson GN, Richards CS, Katz K, and Brookshire GS (1992) Non-specific X linked mental retardation with aphasia exhibiting genetic linkage to chromosomal region Xp11. *J Med Genet* **29**: 629-634.
36. Glass IA, White EM, Pope MJ, Pirrit LA, Cockburn F, and Connor JM (1991) Linkage analysis in a large family with nonspecific X-linked mental retardation. *Am J Med Genet* **38**: 240-243.
37. Claes S, Gu XX, Legius E, Lorenzetti E, Marynen P, Fryns JP, Cassiman JJ, and Raeymaekers P (1996) Linkage analysis in three families with nonspecific X-linked mental retardation. *Am J Med Genet* **64**: 137-146.
38. Bar-David S, Lerer I, Sarfaty CK, Kohan ZG, Meiner V, Zlotogora J, and Abeliovich D (1996) Localization of two X-linked mental retardation (XLMR) genes to Xp: MRX37 gene at Xp22.31-p22.32 and a putative MRX gene on Xp22.11-p22.2. *Am J Med Genet* **64**: 83-88.
39. Bienvenu T, des Portes V, Saint Martin A, McDonell N, Billuart P, Carrié A, Vinet M-C, Couvert P, Toniolo D, Ropers H-H et al (1998) Non-specific X-linked semidominant mental retardation by mutations in a Rab GDP-dissociation inhibitor. *Hum Mol Genet* **7**: 1311-1315.
40. D'Adamo P, Menegon A, Lo Nigro C, Grasso M, Gulisano M, Tamanini F, Bienvenu T, Gedeon AK, Oostra B, Wu S-K et al (1998) Mutations in *GDI1* are responsible for X-linked non-specific mental retardation. *Nature Genet* **19**: 134-139.
41. Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC, Zemni R, Roest Crollius H, Carrié A, Fauchereau F, Cherry M et al (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**: 923-926.
42. Allen KM, Gleeson JG, Bagrodia S, Partington MW, MacMillan JC, Cerione RA, Mulley JC, and Walsh CA (1998) *PAK3* mutation in nonsyndromic X-linked mental retardation. *Nat Genet* **20**: 25-30.
43. Bienvenu T, des Portes V, McDonell N, Carrié A, Zemni R, Couvert P, Ropers HH, Moraine C, van Bokhoven H, Fryns JP et al (2000) Missense mutation in PAK3, R67C, causes X-linked nonspecific mental retardation. *Am J Med Genet* **93**: 294-298.
44. Merienne K, Jacquot S, Pannetier S, Zeniou M, Bankier A, Gecz J, Mandel J-L, Mulley J, Sassone-Corsi P, and Hanauer A (1999) A missense mutation in *RPS6KA3* (*RSK2*) responsible for non-specific mental retardation. *Nat Genet* **22**: 13-14.

45. Carrié A, Jun L, Bienvenu T, Vinet M-C, McDonell N, Couvert P, Zemni R, Cardona A, Van Buggenhout G, Frints S et al (1999) A new member of the IL-1 receptor family highly expressed in hippocampus and involved in X-linked mental retardation. *Nat Genet* **23**: 25-31.
46. Couvert P, Bienvenu T, Aquaviva C, Poirier K, Moraine C, Gendrot C, Verloes A, Andrès C, Le Fevre AC, Souville I et al (2001) *MECP2* is highly mutated in X-linked mental retardation. *Hum Mol Genet* **10**: 941-946.
47. Gecz J, Gedeon AK, Sutherland GR, and Mulley JC (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation. *Nat Genet* **13**: 105-108.
48. Gu Y, Shen Y, Gibbs RA, and Nelson DL (1996) Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island. *Nat Genet* **13**: 109-113.
49. Zemni R, Bienvenu T, Vinet MC, Sefiani A, Carrié A, Billuart P, McDonell N, Couvert P, Francis F, Chafey P et al (2000) A new gene involved in X-linked mental retardation identified by analysis of an X;2 balanced translocation. *Nat Genet* **24**: 167-170.
50. Kerr B, Gedeon A, Mulley J, and Turner G (1992) Localization of non-specific X linked mental retardation genes. *Am J Med Genet* **43**: 392-401.
51. Gedeon AK, Donnelly AJ, Mulley JC, Kerr B, and Turner G (1996) How many X-linked genes for non-specific mental retardation (MRX) are there? *Am J Med Genet* **64**: 158-162.
52. Arveiler B, Alembik Y, Hanauer A, Jacobs P, Tranebjaerg L, Mikkelsen M, Puissant H, Larget Piet L, and Mandel JL (1988) Linkage analysis suggests at least two loci for X-linked non-specific mental retardation. *Am J Med Genet* **30**: 473-483.
53. Hu L-J, Blumenfeld-Heyberger S, Hanauer A, Weissenbach J, and Mandel J-L (1994) Non-specific X-linked mental retardation: linkage analysis in MRX2 and MRX4 families revisited. *Am J Med Genet* **51**: 569-574.
54. Gedeon A, Kerr B, Mulley J, and Turner G (1991) Localisation of the MRX3 gene for non-specific X linked mental retardation. *J Med Genet* **28**: 372-377.
55. Samanns C, Albrecht R, Neugebauer M, Neri G, and Gal A (1991) Gene for non-specific X-linked mental retardation maps in the pericentromeric region. *Am J Med Genet* **38**: 224-227.
56. Kondo I, Tsukamoto K, Niikawa N, Okano K, Kanazawa I, and Hupkes PE (1991) A new form of X-linked mental retardation (XLMR) linked to DXS369(RN1). *Cytogenet Cell Genet* **58**: 2071.
57. Jedele KB, Michels VV, Schaid DJ, Schowalter KV, and Thibodeau SN (1992) Linkage of nonspecific X-linked mental retardation to Xq21.31. *Am J Med Genet* **43**: 436-442.
58. Tackels D, Schwartz CE, Thibodeau SN, and Michels VV (1999) Refined gene localization for MRX7. *Am J Med Genet* **85**: 288-289.
59. Schwartz CE, May M, Huang T, Ledbetter D, Anderson G, Barker DF, Lubs HA, Arena F, and Stevenson RE (1992) MRX8: an X-linked mental retardation condition with linkage to Xq21. *Am J Med Genet* **43**: 467-474.
60. Tackels D, and Schwartz CE (1999) Refined gene localization for MRX8. *Am J Med Genet* **85**: 309-310.
61. Willems P, Vits L, Buntinx I, Raeymaekers P, Van Broeckhoven C, and Ceulemans B (1993) Localization of a gene responsible for nonspecific mental retardation (MRX9) to the pericentromeric region of the X chromosome. *Genomics* **18**: 290-294.
62. Gendrot C, Ronce N, Toutain A, Moizard MP, Muh JP, Raynaud M, Dourlens J, Briault S, and Moraine C (1994) X-linked mental retardation exhibiting linkage to DXS255 and PGKP1: a new MRX family (MRX14) with localization in the pericentromeric region. *Clin Genet* **45**: 145-153.
63. Raynaud M, Gendrot C, Dessay B, Moncla A, Ayrault A-D, Moizard M-P, Toutain A, Briault S, Villard L, Ronce N, and Moraine C (1996) X-linked mental retardation with neonatal hypotonia in a French family (MRX15): gene assignment to Xp11.22-Xp21.1. *Am J Med Genet* **64**: 97-106.
64. Lazzarini A, Stenroos ES, Lehner T, McKoy V, Gold B, McCormack MK, Reid CS, Ott J, and Johnson WG (1995) Short tandem repeat polymorphism linkage studies in a new family with X-linked mental retardation (MRX20). *Am J Med Genet* **57**: 552-557.
65. Kozak L, Chiurazzi P, Genuardi M, Pomponi MG, Zollino M, and Neri G (1993) Mapping of a gene for non-specific X linked mental retardation: evidence for linkage to chromosomal region Xp21.1-Xp22.3. *J Med Genet* **30**: 866-869.
66. Passos-Bueno MR, Byth BC, Rosenberg S, Takata RI, Bakker E, Beggs AH, Pavanello RC, Vainzof M, Davies KE, and Zatz M (1993) Severe nonspecific X-linked mental retardation caused by a proximally Xp located gene: intragenic heterogeneity or a new form of X-linked mental retardation? *Am J Med Genet* **46**: 172-175.
67. Gregg RG, Palmer C, Kirkpatrick S, and Simantel A (1996) Localization of a non-specific X-linked mental retardation gene, MRX23, to Xq23-q24. *Hum Mol Genet* **5**: 411-414.
68. Martinez F, Gal A, Palau F, and Prieto F (1995) Localization of a gene for X-linked nonspecific mental retardation (MRX24) in Xp22.2-p22.3. *Am J Med Genet* **55**: 387-390.
69. Nordström A-M, Penttinen M, and von Koskull H (1992) Linkage to Xq28 in a family with nonspecific X-linked mental retardation. *Hum Genet* **90**: 263-266.
70. Robledo R, Melis P, Laficara F, Marchi J, Rinaldi A, Siniscalco M, and Filippi G (1996) Further linkage evidence for localization of mutational sites for nonsyndromic types of X-linked mental retardation at the pericentromeric region. *Am J Med Genet* **64**: 107-112.
71. Gedeon AK, Glass IA, Connor JM, and Mulley JC (1996) Genetic localisation of MRX27 to Xq24-26 defines another discrete gene for non-specific X-linked mental retardation. *Am J Med Genet* **64**: 121-124.
72. Holinski-Feder E, Golla A, Rost I, Seidel H, Rittinger O, and Meindl A (1996) Regional localization of two MRX genes to Xq28 (MRX28) and to Xp11.4- Xp22.12 (MRX33). *Am J Med Genet* **64**: 125-130.
73. Häne B, Schroer RJ, Arena JF, Lubs HA, Schwartz CE, and Stevenson RE (1996) Nonsyndromic X-linked mental retardation: review and mapping of MRX29 to Xp21. *Clin Genet* **50**: 176-183.
74. Donnelly AJ, Partington MW, Ryan AK, and Mulley JC (1996) Regional localisation of two non-specific X-linked mental retardation genes (*MRX30* and *MRX31*). *Am J Med Genet* **64**: 113-120.
75. Häne B, Stevenson RE, Arena JF, Lubs HA, Simensen RJ, and Schwartz CE (1999) Gene for apparently nonsyndromic X-linked mental retardation (MRX32) maps to an 18-Mb region of Xp21.2-p22. *Am J Med Genet* **85**: 271-275.
76. Gu XX, Decorte R, Marynen P, Fryns J-P, Cassiman J-J, and Raeymaekers P (1996) Localisation of a new gene for non-specific mental retardation to Xq22-q26 (MRX35). *J Med Genet* **33**: 52-55.
77. Schutz CK, Ives EJ, Chalifoux M, MacLaren L, Farrell S, Robinson PD, White BN, and Holden JJA (1996) Regional localization of an X-linked mental retardation gene to Xp21.1- Xp22.13 (MRX38). *Am J Med Genet* **64**: 89-96.
78. Teboul M, Mujica P, Chery M, Leotard B, and Gilgenkrantz S (1989) Balanced X-autosomal translocation and mental retardation. Mapping mental retardation linked to X (excluding fragile X). *J Genet Hum* **37**: 179-195.
79. May M, Colleaux L, Murgia A, Aylsworth A, Nussbaum R, Fontes M, and Schwartz C (1995) Molecular analysis of four males with mental retardation and deletions of Xq21 places the putative MR region in Xq21.1 between DXS233 and CHM. *Hum Mol Genet* **4**: 1465-1466.
80. Holinski-Feder E, Charockh-Zadeh S, Golla A, Rittinger O, Seidel H, Mbert A, Seemanova E, Murken J, and Meindl A (1997) Non-syndromic X-linked mental retardation: mapping of four novel MRX families. *Eighth international workshop on Fragile X and X-linked mental retardation, Picton, Canada.*

81. Hamel BCJ, Smits APT, van den Helm B, Smeets DFCM, Knoers NVAM, van Roosmalen T, Thoonen GHJ, Assman-Hulsmans CFCH, Ropers H-H, Mariman ECM et al (1999) Four families (MRX43, MRX44, MRX45, MRX52) with nonspecific X-linked mental retardation: clinical and psychometric data and results of linkage analysis. *Am J Med Genet* **85**: 290-304.
82. Claes S, Vogels A, Holvoet M, Devriendt K, Raeymaekers P, Cassiman JJ, and Fryns JP (1997) Regional localization of two genes for nonspecific X-linked mental retardation to Xp22.3-p22.2 (MRX49) and Xp11.3-p11.21 (MRX50). *Am J Med Genet* **73**: 474-479.
83. Claes S, Volcke P, Devriendt K, Holvoet M, Raeymaekers P, Cassiman J-J, and Fryns J-P (1999) Regional localization of a gene for nonspecific XLMR to Xp11.3-p11. 23 (MRX51) and tentative localization of an MRX gene to Xq23-q26.1. *Am J Med Genet* **85**: 283-287.
84. Ahmad W, Noci S, ul Haque MF, Sarno T, Aridon P, Ahmad MM, Amin-Ud-Din M, Rafiq MA, ul Haque S, De Fusco M et al (2001) Linkage mapping of a nonspecific form of X-linked mental retardation (MRX53) in a large Pakistani family. *Am J Med Genet* **100** : 62-65.
85. Jemaa LB, des Portes V, Zemni R, Mrad R, Maazoul F, Beldjord C, Chaabouni H, and Chelly J (1999) Refined 2.7 centimorgan locus in Xp21.3-22.1 for a nonspecific X-linked mental retardation gene (MRX54). *Am J Med Genet* **85**: 276-282.
86. Deqaqi SC, N'Guessan M, Forner J, Sbiti A, Beldjord C, Chelly J, Sefiani A, and des Portes V (1998) A gene for non-specific X-linked mental retardation (MRX55) is located in Xp11. *Ann Genet* **41**: 11-16.
87. Holinski-Feder E, Chahrockh-Zadeh S, Rittinger O, Jedele KB, Gasteiger M, Lenski C, Murken J, and Golla A (1999) Nonsyndromic X-linked mental retardation: mapping of MRX58 to the pericentromeric region. *Am J Med Genet* **86**: 102-106.
88. Carpenter NJ, Brown WT, Qu Y, and Keenan KL (1999) Regional localization of a nonspecific X-linked mental retardation gene (MRX59) to Xp21.2-p22.2. *Am J Med Genet* **85**: 266-270.
89. Pai GS, Hane B, Joseph M, Nelson R, Hammond LS, Arena JF, Lubs HA, Stevenson RE, and Schwartz CE (1997) A new X linked recessive syndrome of mental retardation and mild dysmorphism maps to Xq28. *J Med Genet* **34**: 529-534.
90. Frints SGM, Claes S, Gooris D, Volcke P, Swinnen L, Borghgraef M, Devriendt K, van den Berghe L, Holvoet M, Marynen P, Cassiman JJ, and Fryns J-P (1999) Linkage analysis in four families (MRX68, MRX69, MRX70, MRX71) with non-specific X-linked mental retardation. *9th International Workshop on Fragile X Syndrome and X Linked Mental Retardation, Strasbourg, France.*
91. Russo S, Cogliati F, Cavalleri F, Cassitto MG, Giglioli R, Toniolo D, Casari G, and Larizza L (2000) Mapping to distal Xq28 of nonspecific X-linked mental retardation MRX72: Linkage analysis and clinical findings in a three-generation Sardinian family. *Am J Med Genet* **94**: 376-382.
92. Caspari R, Uhlhaas S, Friedl W, Knapp M, and Propping P (2000) Mapping of a gene for nonspecific X-linked mental retardation (MRX 75) to Xq24-q26. *Am J Med Genet* **93** : 290-293.
93. Nelson DL, Ballabio A, Cremers F, Monaco AP, and Schlessinger D (1995) Report of the sixth international workshop on X chromosome mapping 1995. *Cytogenet Cell Genet* **71**: 307-342.
94. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, and et al (2001) The sequence of the human genome. *Science* **291**: 1304-1351.
95. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W et al (2001) Initial sequencing and analysis of the human genome. *Nature* **409**: 860-921.
96. Muroya K, Kinoshita E, Kamimaki T, Matsuo N, Yorifugi T, and Ogata T (1999) Deletion mapping and X inactivation analysis of a non-specific mental retardation gene at Xp21.3-Xp22.11. *J Med Genet* **36**: 187-191.
97. Bach I, Robinson D, Thomas N, Ropers H-H, and Cremers FPM (1992) Physical fine mapping of genes underlying X-linked deafness and non fra(X)-X-linked mental retardation at Xq21. *Hum Genet* **89**: 620-624.
98. Huber I, Bitner-Glindzicz M, de Kok YJM, van der Maarel SM, Ishikawa-Brush Y, Monaco AP, Robinson D, Malcolm S, Pembrey ME, Brunner HG et al (1994) X-linked mixed deafness (DFN3): cloning and characterization of the critical region allows the identification of novel microdeletions. *Hum Mol Genet* **3**: 1151-1154.
99. McCabe ERB, Towbin JA, van den Engh G, and Trask BJ (1992) Xp21 contiguous gene syndromes: deletion quantitation with bivariate flow karyotyping allows mapping of patient breakpoints. *Am J Hum Genet* **51**: 1277-1285.
100. Billuart P, Vinet MC, des Portes V, Llense S, Richard L, Moutard ML, Recan D, Brüls T, Bienvenu T, Kahn A, Beldjord C, and Chelly J (1996) Identification by STS PCR screening of a microdeletion in Xp21.3-22.1 associated with non-specific mental retardation. *Hum Mol Genet* **5**: 977-979.
101. Raeymaekers P, Lin J, Gu XX, Soekarman D, Cassiman J-J, Fryns J-P, and Marynen P (1996) A form of non-specific mental retardation is probably caused by a microdeletion in a Belgian family. *Am J Med Genet* **64**: 16.
102. Gécz J, Oostra BA, Hockey A, Carbonell P, Turner G, Haan EA, Sutherland GR, and Mulley JC (1997) FMR2 expression in families with *FRAXE* mental retardation. *Hum Mol Genet* **6**: 435-441.
103. Gedeon AK, Meinanen M, Ades LC, Kaariainen H, Gecz J, Baker E, Sutherland GR, and Mulley JC (1995) Overlapping submicroscopic deletions in Xq28 in two unrelated boys with developmental disorders: identification of a gene near FRAXE. *Am J Hum Genet* **56**: 907-914.
104. Fries MH, Lebo RV, Schonberg SA, Golabi M, Seltzer WK, Gitelman SE, and Golbus MS (1993) Mental retardation locus in Xp21 chromosome microdeletion. *Am J Med Genet* **46**: 363-368.
105. des Portes V, Carrié A, Billuart P, Kieffer V, Bienvenu T, Vinet MC, Beldjord C, Kahn A, Ponsot G, Chelly J et al (1998) Inherited microdeletion in Xp21.3-22.1 involved in non-specific mental retardation. *Clin Genet* **53**: 136-141.
106. van der Maarel SM, Scholten IHJM, Huber I, Philippe C, Suijkerbuijk RF, Gilgenkrantz S, Kere J, Cremers FPM, and Ropers H-H (1996) Cloning and characterization of *DXS6673E*, a candidate gene for X-linked mental retardation in Xq13.1. *Hum Mol Genet* **5**: 887-897.
107. Gécz J, Barnett S, Liu J, Hollway G, Donnelly A, Eyre H, Eshkevari HS, Baltazar R, Grunn A, Nagaraja R et al. (1999) Characterization of the human glutamate receptor subunit 3 gene (*GRIA3*), a candidate for bipolar disorder and nonspecific X-linked mental retardation. *Genomics* **62**: 356-368.
108. Fukami M, Kirsch S, Schiller S, Richter A, Benes V, Franco B, Muroya K, Rao E, Merker S, Niesler B et al (2000) A member of a gene family on Xp22.3, *VCX-A*, is deleted in patients with X-linked nonspecific mental retardation. *Am J Hum Genet* **67**: 563-573.
109. Nothwang HG, Schroer A, van der Maarel S, Kubart S, Schneider S, Riesselmann L, Menzel C, Hinzmann B, Vogt D, Rosenthal A, Fryns J, Tommerup N, Haaf T, Ropers HH, Wirth J. (2000) Molecular cloning of Xp11 breakpoints in two unrelated mentally retarded females with X;autosome translocations. *Cytogenet Cell Genet* **90**: 126-133.
110. Kleinjan D-J, and van Heyningen V (1998) Position effect in human genetic disease. *Hum Mol Genet* **7**: 1611-1618.
111. de Kok YJM, van der Maarel SM, Bitner-Glindzicz M, Huber I, Monaco AP, Malcolm S, Pembrey ME, Ropers H-H, and Cremers FPM (1995) Association between X-linked mixed deafness and mutations in the POU domain gene *POU3F4*. *Science* **267**: 685-688.
112. de Kok YJM, Merkx GF, van der Maarel SM, Huber I, Malcolm S, Ropers HH, and Cremers FPM (1995) A duplication/paracentric inversion associated with familial X-linked deafness (DFN3) suggests the presence of a regulatory element more than 400 kb upstream of the *POU3F4* gene. *Hum Mol Genet* **4**: 2145-2150.

113. de Kok YJM, Vossenaar ER, Cremers CWRJ, Dahl N, Laporte J, Hu LJ, Lacombe D, Fischel-Ghodsian N, Friedman RA, Parnes LS et al (1996) Identification of a hot spot for microdeletions in patients with X-linked deafness type 3 (DFN3) 900 kb proximal to the DFN3 gene *POU3F4*. *Hum Mol Genet* **5**: 1229-1235.
114. Cremers FPM, Cremers CW, and Ropers H-H. (2000) The ins and outs of X-linked deafness type 3. *Adv Otorhinolaryngol* **56**: 184-195.
115. Ballabio A (1993) The rise and fall of positional cloning? *Nat Genet* **3**: 277-279.
116. Fischer von Mollard G, Stahl B, Khokhlatchev A, Südhof TC, and Jahn R (1994) Rab3C is a synaptic vesicle protein that dissociates from synaptic vesicles after stimulation of exocytosis. *J Biol Chem* **269**: 10971-10974.
117. Fischer von Mollard G, Stahl B, Li C, Südhof TC, and Jahn R (1994) Rab proteins in regulated exocytosis. *Trends Biochem Sci* **19**: 164-168.
118. Wang Y, Okamoto M, Schmitz F, Hofmann K, and Südhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. *Nature* **388**: 593-598.
119. Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Hammer RE, and Südhof TC (1994) The role of Rab3A in neurotransmitter release. *Nature* **369**: 493-497.
120. Takai Y, Sasaki T, Shirataki H, and Nakanishi H (1996) Rab3A small GTP-binding protein in Ca(2+)-dependent exocytosis. *Genes Cells* **1**: 615-632.
121. Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, and Walsh CA (1998) *Doublecortin*, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* **92**: 63-72.
122. Allen KM, Gleeson JG, Shoup SM, and Walsh CA (1998) A YAC contig in Xq22.3-q23, from *DXS287* to *DXS8088*, spanning the brain-specific genes *doublecortin* (*DCX*) and *PAK3*. *Genomics* **52**: 214-218.
123. Manouvrier-Hanu S, Amiel J, Jacquot S, Merienne K, Moerman A, Coëslier A, Labarriere F, Vallée L, Croquette MF, and Hanauer A (1999) Unreported *RSK2* missense mutation in two male sibs with an unusually mild form of Coffin-Lowry syndrome. *J Med Genet* **36**: 775-778.
124. Orrico A, Lam C-W, Galli L, Dotti MT, Hayek G, Tong S-F, Poon PMK, Zappella M, Federico A, and Sorrentino V (2000) *MECP2* mutation in male patients with non-specific X-linked mental retardation. *FEBS Lett* **481**: 285-288.
125. Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, and Zoghbi HY (1999) Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. *Nat Genet* **23**: 185-188.
126. Gibbons RJ, Picketts DJ, Villard L, and Higgs DR (1995) Mutations in a putative global transcriptional regulator cause X-linked mental retardation with α-thalassemia (ATR-X syndrome). *Cell* **80**: 837-845.
127. Villard L, Gecz J, Mattéi JF, Fontés M, Saugier-Veber P, Munnich A, and Lyonnet S (1996) XNP mutation in a large family with Juberg-Marsidi syndrome. *Nature Genet* **12**: 359-360.
128. Abidi F, Schwartz CE, Carpenter NJ, Villard L, Fontés M, and Curtis M (1999) Carpenter-Waziri syndrome results from a mutation in XNP. *Am J Med Genet* **85**: 249-251.
129. Lossi AM, Millán JM, Villard L, Orellana C, Cardoso C, Prieto F, Fontés M, and Martínez F (1999) Mutation of the XNP/ATR-X gene in a family with severe mental retardation, spastic paraplegia and skewed pattern of X inactivation: demonstration that the mutation is involved in the inactivation bias. *Am J Hum Genet* **65**: 558-562.
130. Stevenson RE, Abidi F, Schwartz CE, Lubs HA, and Holmes LB (2000) Holmes-gang syndrome is allelic with XLMR-hypotonic face syndrome. *Am J Med Genet* **94**: 383-385.
131. Villard L, Fontès M, Adès LC, and Gecz J (2000) Identification of a mutation in the *XNP/ATR-X* gene in a family reported as Smith-Fineman-Myers syndrome. *Am J Med Genet* **91**: 83-85.
132. Guerrini R, Shanahan JL, Carrozzo R, Bonanni P, Higgs DR, and Gibbons RJ (2000) A nonsense mutation of the *ATRX* gene causing mild mental retardation and epilepsy. *Ann Neurol* **47**: 117-121.
133. Brunner HG, Nelen M, Breakefield XO, Ropers HH, and van Oost BA (1993) Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. *Science* **262**: 578-580.
134. Pasteris NG, Cadle A, Logie LJ, Porteous ME, Schwartz CE, Stevenson RE, Glover TW, Wilroy RS, and Gorski JL (1994) Isolation and characterization of the faciogenital dysplasia (Aarskog- Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. *Cell* **79**: 669-678.
135. Berger W, van de PD, Warburg M, Gal A, Bleeker-Wagemakers L, de Silva H, Meindl A, Meitinger T, Cremers F, and Ropers HH (1992) Mutations in the candidate gene for Norrie disease. *Hum Mol Genet* **1**: 461-465.
136. Tanaka E, and Sabry J (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* **83**: 171-176.
137. Goldberg DJ, and Burmeister DW (1986) Stages in axon formation: observations of growth of Aplysia axons in culture using video-enhanced contrast-differential interference contrast microscopy. *J Cell Biol* **103**: 1921-1931.
138. Letourneau PC (1983) Differences in the organization of actin in the growth cones compared with the neurites of cultured neurons from chick embryos. *J Cell Biol* **97** : 963-973.
139. Gordon-Weeks PR (1993) Organization of microtubules in axonal growth cones: a role for microtubule-associated protein MAP 1B. *J Neurocytol* **22**: 717-725.
140. Nobes CD, and Hall A (1995) Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* **23**: 456-459.
141. Nobes CD, and Hall A (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**: 53-62.
142. Vancura KL, and Jay DG (1998) G Proteins and axon growth. *seminars in Neurosc* **9**: 209-219.
143. Bridgman PC, and Dailey ME (1989) The organization of myosin and actin in rapid frozen nerve growth cones. *J Cell Biol* **108**: 95-109.
144. Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, and Jan YN (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**: 837-840.
145. Zipkin ID, Kindt RM, and Kenyon CJ (1997) Role of a new Rho family member in cell migration and axon guidance in C. elegans. *Cell* **90**: 883-894.
146. Threadgill R, Bobb K, and Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron* **19**: 625-634.
147. Kandel ER, Schwartz JH, and Jessell TM (1991) Principles of neural science, 3rd edition. New York: Elsevier Science Publishing.
148. Squire LR (1986) Mechanisms of memory. *Science* **232**: 1612-1619.
149. Bliss TV, and Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* **232**: 331-356.
150. Matus A (2000) Actin-based plasticity in dendritic spines. *Science* **290**: 754-758.
151. Bear MF (1997) How do memories leave their mark? *Nature* **385**: 481-482.
152. Bliss TVP, and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**: 31-39.
153. Yin JC, and Tully T (1996) CREB and the formation of long-term memory. *Curr Opin Neurobiol* **6**: 264-268.
154. Purpura DP (1974) Dendritic spine "dysgenesis" and mental retardation. *Science* **186**: 1126-1128.
155. Purpura DP, Bodick N, Suzuki K, Rapin I, and Wurzelmann S (1982) Microtubule disarray in cortical dendrites and neurobehavioral failure. I. Golgi and electron microscopic studies. *Brain Res* **281**: 287-297.

156. Rudelli RD, Brown WT, Wisniewski K, Jenkins EC, Laure-Kamionowska M, Connell F, and Wisniewski HM (1985) Adult fragile X syndrome. Clinico-neuropathologic findings. *Acta Neuropathol (Berl)* **67**: 289-295.
157. Hinton VJ, Brown WT, Wisniewski K, and Rudelli RD (1991) Analysis of neocortex in three males with the fragile X syndrome. *Am J Med Genet* **41**: 289-294.
158. Huttenlocher PR (1974) Dendritic development in neocortex of children with mental defect and infantile spasms. *Neurology* **24**: 203-210.
159. Marin-Padilla M (1976) Pyramidal cell abnormalities in the motor cortex of a child with Down's syndrome. A Golgi study. *J Comp Neurol* **167**: 63-81.
160. Williams RS, Hauser SL, Purpura DP, DeLong GR, and Swisher CN (1980) Autism and mental retardation: neuropathologic studies performed in four retarded persons with autistic behavior. *Arch Neurol* **37**: 749-753.
161. Aridor M, and Balch WE (1996) Principles of selective transport: coat complexes hold the key. *Trends Cell Biol* **6**: 315-320.
162. Boguski MS, and McCormick F (1993) Proteins regulating Ras and its relatives. *Nature* **366**: 643-654.
163. Farnsworth CC, Kawata M, Yoshida Y, Takai Y, Gelb MH, and Glomset JA (1991) C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. *Proc Natl Acad Sci USA* **88**: 6196-6200.
164. Seabra MC, Goldstein JL, Sudhof TC, and Brown MS (1992) Rab geranylgeranyl transferase. A multisubunit enzyme that prenylates GTP-binding proteins terminating in Cys-X-Cys or Cys-Cys. *J Biol Chem* **267**: 14497-14503.
165. Andres DA, Seabra MC, Brown MS, Armstrong SA, Smeland TE, Cremers FPM, and Goldstein JL (1993) cDNA cloning of component A of Rab geranylgeranyl transferase and demonstration of its role as a Rab escort protein. *Cell* **73**: 1091-1099.
166. Bächner D, Sedlacek Z, Korn B, Hameister H, and Poustka A (1995) Expression patterns of two human genes coding for different *rab* GDP- dissociation inhibitors (GDIs), extremely conserved proteins involved in cellular transport. *Hum Mol Genet* **4**: 701-708.
167. Sasaki T, Kikuchi A, Araki S, Hata Y, Isomura M, Kuroda S, and Takai Y (1990) Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to *smg* p25A, a *ras* p21-like GTP-binding protein. *J Biol Chem* **265**: 2333-2337.
168. Wu S-K, Zeng K, Wilson IA, and Balch WE (1996) Structural insights into the function of the Rab GDI superfamily. *Trends Biochem Sci* **21**: 472-476.
169. Pfeffer SR, Dirac-Svejstrup AB, and Soldati T (1995) Rab GDP dissociation inhibitor: putting rab GTPases in the right place. *J Biol Chem* **270**: 17057-17059.
170. Cremers FP, Armstrong SA, Seabra MC, Brown MS, and Goldstein JL (1994) REP-2, a Rab escort protein encoded by the choroideremia-like gene. *J Biol Chem* **269**: 2111-2117.
171. Seabra MC, Ho YK, and Anant JS (1995) Deficient geranylgeranylation of Ram/Rab27 in choroideremia. *J Biol Chem* **270**: 24420-24427.
172. Seabra MC, Brown MS, Slaughter CA, Sudhof TC, and Goldstein JL (1992) Purification of component A of Rab geranylgeranyl transferase: possible identity with the choroideremia gene product. *Cell* **70**: 1049-1057.
173. Leoni C, Menegon A, Benfenati F, Toniolo D, Pennuto M, and Valtorta F (1999) Neurite extension occurs in the absence of regulated exocytosis in PC12 subclones. *Mol Biol Cell* **10**: 2919-2931.
174. Ishizaki H, Miyoshi J, Kamiya H, Togawa A, Tanaka M, Sasaki T, Endo K, Mizoguchi A, Ozawa S, and Takai Y (2000) Role of rab GDP dissociation inhibitor α in regulating plasticity of hippocampal neurotransmission. *Proc Natl Acad Sci USA* **97**: 11587-11592.
175. Bienvenu T, Der-Sarkissian H, Billuart P, Tissot M, des Portes V, Brüls T, Chabrolle J-P, Chauveau P, Cherry M, Kahn A et al (1997) Mapping of the X-breakpoint involved in a balanced X;12 translocation in a female with mild mental retardation. *Eur J Hum Genet* **5**: 105-109.
176. Billuart P, Chelly J, Carrie A, Vinet M-C, Couvert P, McDonell N, Zemni R, Kahn A, Moraine C, Beldjord C et al (2000) Determination of the gene structure of human *oligophrenin-1* and identification of three novel polymorphisms by screening of DNA from 164 patients with non-specific X-linked mental retardation. *Ann Genet* **43**: 5-9.
177. Van Aelst L, and D'Souza-Schorey C (1997) Rho GTPases and signaling networks. *Genes Dev* **11**: 2295-2322.
178. Chang JH, Gill S, Settleman J, and Parsons SJ (1995) c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. *J Cell Biol* **130**: 355-368.
179. Diekmann D, Brill S, Garrett MD, Totty N, Hsuan J, Monfries C, Hall C, Lim L, and Hall A (1991) Bcr encodes a GTPase-activating protein for p21rac. *Nature* **351**: 400-402.
180. Manser E, Chong C, Zhao ZS, Leung T, Michael G, Hall C, and Lim L (1995) Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family. *J Biol Chem* **270**: 25070-25078.
181. Kozma R, Ahmed S, Best A, and Lim L (1996) The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Mol Cell Biol* **16**: 5069-5080.
182. Settleman J, Albright CF, Foster LC, and Weinberg RA (1992) Association between GTPase activators for Rho and Ras families. *Nature* **359**: 153-154.
183. Foster R, Hu KQ, Shaywitz DA, and Settleman J (1994) p190 RhoGAP, the major RasGAP-associated protein, binds GTP directly. *Mol Cell Biol* **14**: 7173-7181.
184. Ljubimova JY, Khazenzon NM, Chen Z, Neyman YI, Turner L, Riedinger MS, and Black KL (2001) Gene expression abnormalities in human glial tumors identified by gene array. *Int J Oncol* **18**: 287-295.
185. Manser E, Leung T, Salihuddin H, Zhao ZS, and Lim L (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**: 40-46.
186. Knaus UG, Morris S, Dong HJ, Chernoff J, and Bokoch GM (1995) Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science* **269**: 221-223.
187. Brown JL, Stowers L, Baer M, Trejo J, Coughlin S, and Chant J (1996) Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. *Curr Biol* **6**: 598-605.
188. Teo M, Manser E, and Lim L (1995) Identification and molecular cloning of a p21cdc42/rac1-activated serine/ threonine kinase that is rapidly activated by thrombin in platelets. *J Biol Chem* **270**: 26690-26697.
189. Jakobi R, Chen CJ, Tuazon PT, and Traugh JA (1996) Molecular cloning and sequencing of the cytostatic G protein-activated protein kinase PAK I. *J Biol Chem* **271**: 6206-6211.
190. Bagrodia S, Taylor SJ, Creasy CL, Chernoff J, and Cerione RA (1995) Identification of a mouse $p21^{Cdc42/Rac}$ activated kinase. *J Biol Chem* **270**: 22731-22737.
191. Abo A, Qu J, Cammarano MS, Dan C, Fritsch A, Baud V, Belisle B, and Minden A (1998) PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia. *EMBO J* **17**: 6527-6540.
192. Sells MA, and Chernoff J (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol* **7**: 162-167.
193. Lim L, Manser E, Leung T, and Hall C (1996) Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras- related Rho subfamily and its role in phosphorylation signalling pathways. *Eur J Biochem* **242**: 171-185.
194. Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GM, and Chernoff J (1997) Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* **7**: 202-210.
195. Daniels RH, Hall PS, and Bokoch GM (1998) Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. *EMBO J* **17**: 754-764.

196. Manser E, Loo T-H, Koh C-G, Zhao Z-S, Chen X-Q, Tan L, Tan I, Leung T, and Lim L (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell* **1**: 183-192.
197. Obermeier A, Ahmed S, Manser E, Yen SC, Hall C, and Lim L (1998) PAK promotes morphological changes by acting upstream of Rac. *EMBO J* **17**: 4328-4339.
198. Kozma R, Sarner S, Ahmed S, and Lim L (1997) Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol Cell Biol* **17**: 1201-1211.
199. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenstrom P, Bridges T, Chant J, and Hall A (1996) Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell* **87**: 519-529.
200. Joneson T, McDonough M, Bar-Sagi D, and Van Aelst L (1996) RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science* **274**: 1374-1376.
201. Catling AD, Schaeffer HJ, Reuter CW, Reddy GR, and Weber MJ (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. *Mol Cell Biol* **15**: 5214-5225.
202. Frost JA, Khokhlatchev A, Stippec S, White MA, and Cobb MH (1998) Differential effects of PAK1-activating mutations reveal activity- dependent and -independent effects on cytoskeletal regulation. *J Biol Chem* **273**: 28191-28198.
203. Jelinek T, Catling AD, Reuter CW, Moodie SA, Wolfman A, and Weber MJ (1994) RAS and RAF-1 form a signalling complex with MEK-1 but not MEK-2. *Mol Cell Biol* **14**: 8212-8218.
204. Bagrodia S, Taylor SJ, Jordon KA, Van Aelst L, and Cerione RA (1998) A novel regulator of p21-activated kinases. *J Biol Chem* **273**: 23633-23636.
205. Castresana J, Saraste M (1995) Does Vav bind to F-actin through a CH domain? *FEBS Lett* **374**: 149-151.
206. Zheng Y, Zangrilli D, Cerione RA, and Eva A (1996) The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. *J Biol Chem* **271**: 19017-19020.
207. Horiuchi H, Lippe R, McBride HM, Rubino M, Woodman P, Stenmark H, Rybin V, Wilm M, Ashman K, Mann M, and Zerial M (1997) A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell* **90**: 1149-1159.
208. Oh WK, Yoo JC, Jo D, Song YH, Kim MG, and Park D (1997) Cloning of a SH3 domain-containing proline-rich protein, p85SPR, and its localization in focal adhesion. *Biochem Biophys Res Commun* **235**: 794-798.
209. Maecker HT, Todd SC, and Levy S (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J* **11**: 428-442.
210. Levy S, Todd SC, and Maecker HT (1998) CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* **16**: 89-109.
211. Kitadokoro K, Bordo D, Galli G, Petracca R, Falugi F, Abrignani S, Grandi G, and Bolognesi M (2001) CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. *EMBO J* **20**: 12-18.
212. Hemler ME (1998) Integrin associated proteins. *Curr Opin Cell Biol* **10**: 578-585.
213. Imai T, and Yoshie O (1993) C33 antigen and M38 antigen recognized by monoclonal antibodies inhibitory to syncytium formation by human T cell leukemia virus type 1 are both members of the transmembrane 4 superfamily and associate with each other and with CD4 or CD8 in T cells. *J Immunol* **151**: 6470-6481.
214. Hammond C, Denzin LK, Pan M, Griffith JM, Geuze HJ, and Cresswell P (1998) The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J Immunol* **161**: 3282-3291.
215. Berditchevski F, Zutter MM, and Hemler ME (1996) Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). *Mol Biol Cell* **7**: 193-207.
216. Rubinstein E, Le Naour F, Lagaudriere-Gesbert C, Billard M, Conjeaud H, and Boucheix C (1996) CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur J Immunol* **26**: 2657-2665.
217. Berditchevski F, Tolias KF, Wong K, Carpenter CL, and Hemler ME (1997) A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J Biol Chem* **272**: 2595-2598.
218. Takagi S, Fujikawa K, Imai T, Fukuhara N, Fukudome K, Minegishi M, Tsuchiya S, Konno T, Hinuma Y, and Yoshie O (1995) Identification of a highly specific surface marker of T-cell acute lymphoblastic leukemia and neuroblastoma as a new member of the transmembrane 4 superfamily. *Int J Cancer* **61**: 706-715.
219. Milner B, Squire LR, and Kandel ER (1998) Cognitive neuroscience and the study of memory. *Neuron* **20**: 445-468.
220. Tsien JZ, Huerta PT, and Tonegawa S (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87**: 1327-1338.
221. Huang J, Gao X, Li S, and Cao Z (1997) Recruitment of IRAK to the interleukin 1 receptor complex requires interleukin 1 receptor accessory protein. *Proc Natl Acad Sci U S A* **94**: 12829-12832.
222. Cao Z, Henzel WJ, and Gao X (1996) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**: 1128-1131.
223. Born TL, Smith DE, Garka KE, Renshaw BR, Bertles JS, and Sims JE (2000) Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. *J Biol Chem* **275**: 29946-29954.
224. Sana TR, Debets R, Timans JC, Bazan JF, and Kastelein RA (2000) Computational identification, cloning, and characterization of IL-1R9, a novel interleukin-1 receptor-like gene encoded over an unusually large interval of human chromosome Xq22.2-q22.3. *Genomics* **69**: 252-262.
225. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, and Ju G (1995) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J Biol Chem* **270**: 13757-13765.
226. Wesche H, Resch K, and Martin MU (1998) Effects of IL-1 receptor accessory protein on IL-1 binding. *FEBS Lett* **429**: 303-306.
227. Wesche H, Korherr C, Kracht M, Falk W, Resch K, and Martin MU (1997) The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J Biol Chem* **272**: 7727-7731.
228. Cullinan EB, Kwee L, Nunes P, Shuster DJ, Ju G, McIntyre KW, Chizzonite RA, and Labow MA (1998) IL-1 receptor accessory protein is an essential component of the IL-1 receptor. *J Immunol* **161**: 5614-5620.
229. Muzio M, Ni J, Feng P, and Dixit VM (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL- 1 signaling. *Science* **278**: 1612-1615.
230. Cao Z, Xiong J, Takeuchi M, Kurama T, and Goeddel DV (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383**: 443-446.
231. May MJ, and Ghosh S (1998) Signal transduction through NF-kappa B. *Immunol Today* **19**: 80-88.
232. Born TL, Thomassen E, Bird TA, and Sims JE (1998) Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J Biol Chem* **273**: 29445-29450.
233. Torigoe K, Ushio S, Okura T, Kobayashi S, Taniai M, Kunikata T, Murakami T, Sanou O, Kojima H, Fujii M et al (1997) Purification and characterization of the human interleukin-18 receptor. *J Biol Chem* **272**: 25737-25742.

234. Thomassen E, Bird TA, Renshaw BR, Kennedy MK, and Sims JE (1998) Binding of interleukin-18 to the interleukin-1 receptor homologous receptor IL-1Rrp1 leads to activation of signaling pathways similar to those used by interleukin-1. *J Interferon Cytokine Res* **18**: 1077-1088.
235. Katsuki H, Nakai S, Hirai Y, Akaji K, Kiso Y, and Satoh M (1990) Interleukin-1 beta inhibits long-term potentiation in the CA3 region of mouse hippocampal slices. *Eur J Pharmacol* **181**: 323-326.
236. Rada P, Mark GP, Vitek MP, Mangano RM, Blume AJ, Beer B, and Hoebel BG (1991) Interleukin-1 beta decreases acetylcholine measured by microdialysis in the hippocampus of freely moving rats. *Brain Res* **550**: 287-290.
237. Cassel JC, and Jeltsch H (1995) Serotonergic modulation of cholinergic function in the central nervous system: cognitive implications. *Neuroscience* **69**: 1-41.
238. Sanz MM, Jenkins EC, Brown WT, Davisson MT, Kevin MJ, Roderick TH, Silverman WP, and Wisniewski HM (1986) Mouse chromosome fragility. *Am J Med Genet* **23**: 491-509.
239. Smeets DFCM, and van de Klundert FAJM (1990) Common fragile sites in man and three closely related primate species. *Cytogenet Cell Genet* **53**: 8-14.
240. Wurster-Hill DH, Ward OG, Davis BH, Park JP, Moyzis RK, and Meyne J (1988) Fragile sites, telomeric DNA sequences, B chromosomes, and DNA content in raccoon dogs, Nyctereutes procyonoides, with comparative notes on foxes, coyote, wolf, and raccoon. *Cytogenet Cell Genet* **49**: 278-281.
241. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, and Zhang FP (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* **65**: 905-914.
242. Kremer EJ, Pritchard M, Lynch M, Yu S, Holman K, Baker E, Warren ST, Schlessinger D, Sutherland GR, and Richards RI (1991) Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. *Science* **252**: 1711-1714.
243. Oberle I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas MF, and Mandel JL (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* **252**: 1097-1102.
244. Yu S, Mulley J, Loesch D, Turner G, Donnelly A, Gedeon A, Hillen D, Kremer E, Lynch M, and Pritchard M (1992) Fragile-X syndrome: unique genetics of the heritable unstable element. *Am J Hum Genet* **50**: 968-980.
245. Parrish JE, Oostra BA, Verkerk AJ, Richards CS, Reynolds J, Spikes AS, Shaffer LG, and Nelson DL (1994) Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. *Nat Genet* **8**: 229-235.
246. Jones C, Slijepcevic P, Marsh S, Baker E, Langdon WY, Richards RI, and Tunnacliffe A (1994) Physical linkage of the fragile site FRA11B and a Jacobsen syndrome chromosome deletion breakpoint in 11q23.3. *Hum Mol Genet* **3**: 2123-2130.
247. Nancarrow JK, Holman K, Mangelsdorf M, Hori T, Denton M, Sutherland GR, and Richards RI (1995) Molecular basis of p(CCG)n repeat instability at the FRA16A fragile site locus. *Hum Mol Genet* **4**: 367-372.
248. Knight SJL, Flannery AV, Hirst MC, Campbell L, Christodoulou Z, Phelps SR, Pointon J, Middleton-Price HR, Barnicoat A, Pembrey ME et al (1993) Trinucleotide repeat amplification and hypermethylation of a CpG island in *FRAXE* mental retardation. *Cell* **74**: 127-134.
249. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, and Warren ST (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum Mol Genet* **1**: 397-400.
250. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, and Nelson DL (1991) Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* **66**: 817-822.
251. Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, Holman K, Mulley JC, Warren ST, Schlessinger D et al (1991) Fragile X genotype characterized by an unstable region of DNA. *Science* **252**: 1179-1181.
252. Fu Y-H, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH, Holden JJA, Fenwick RG, Warren ST et al (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* **67**: 1047-1058.
253. Vincent A, Heitz D, Petit C, Kretz C, Oberlé I, and Mandel J-L (1991) Abnormal pattern detected in fragile-X patients by pulsed-field gel electrophoresis. *Nature* **349**: 624-626.
254. Bell MV, Hirst MC, Nakahori Y, MacKinnon RN, Roche A, Flint TJ, Jacobs PA, Tommerup N, Tranebjaerg L, Froster-Iskenius U et al (1991) Physical mapping across the fragile X: hypermethylation and clinical expression of the fragile X syndrome. *Cell* **64**: 861-866.
255. Hansen RS, Gartler SM, Scott CR, Chen S-H, and Laird CD (1992) Methylation analysis of CGG sites in the CpG island of the human *FMR1* gene. *Hum Mol Genet* **1**: 571-578.
256. Sutherland GR, and Baker E (1992) Characterisation of a new rare fragile site easily confused with the fragile X. *Hum Mol Genet* **1**: 111-113.
257. Flynn GA, Hirst MC, Knight SJL, MacPherson JN, Barber JCK, Flannery AV, Davies KE, and Buckle VJ (1993) Identification of the *FRAXE* fragile site in two families ascertained for X linked mental retardation. *J Med Genet* **30**: 97-100.
258. Allingham-Hawkins DJ, and Ray PN (1995) FRAXE expansion is not a common etiological factor among developmentally delayed males. *Am J Hum Genet* **57**: 72-76.
259. Zhong N, Ju W, Curley D, Wang D, Pietrofesa J, Wu G, Shen Y, Pang C, Poon P, Liu X et al (1996) A survey of FRAXE allele sizes in three populations. *Am J Med Genet* **64**: 415-419.
260. Youings SA, Murray A, Dennis N, Ennis S, Lewis C, McKechnie N, Pound M, Sharrock A, and Jacobs P (2000) FRAXA and FRAXE: the results of a five year survey. *J Med Genet* **37**: 415-421.
261. Gecz J, Bielby S, Sutherland GR, and Mulley JC (1997) Gene structure and subcellular localization of FMR2, a member of a new family of putative transcription activators. *Genomics* **44**: 201-213.
262. Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimino G, Croce CM, and Canaani E (1992) The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. *Cell* **71**: 701-708.
263. Ma C, and Staudt LM (1996) LAF-4 encodes a lymphoid nuclear protein with transactivation potential that is homologous to AF-4, the gene fused to MLL in t(4;11) leukemias. *Blood* **87**: 734-745.
264. Taki T, Kano H, Taniwaki M, Sako M, Yanagisawa M, and Hayashi Y (1999) *AF5q31*, a newly identified *AF4*-related gene, is fused to *MLL* in infant acute lymphoblastic leukemia with ins(5;11)(q31;q13q23). *Proc Natl Acad Sci U S A* **96**: 14535-14540.
265. Wittwer F, van der Straten A, Keleman K, Dickson BJ, and Hafen E (2001) Lilliputian: an AF4/FMR2-related protein that controls cell identity and cell growth. *Development* **128**: 791-800.
266. Tang AH, Neufeld TP, Rubin GM, and Müller H-AJ (2001) Transcriptional regulation of cytoskeletal functions and segmentation by a novel maternal pair-rule gene, *lilliputian*. *Development* **128**: 801-813.
267. Mulley JC, Yu S, Loesch DZ, Hay DA, Donnelly A, Gedeon AK, Carbonell P, López I, Glover G, Gabarrón I et al (1995) FRAXE and mental retardation. *J Med Genet* **32**: 162-169.
268. Carbonell P, Lopez I, Gabarron J, Bernabe MJ, Lucas JM, Guitart M, Gabau E, and Glover G (1996) FRAXE mutation analysis in three Spanish families. *Am J Med Genet* **64**: 434-440.
269. Murgia A, Polli R, Vinanzi C, Salis M, Drigo P, Artifoni L, and Zacchello F (1996) Amplification of the Xq28 FRAXE repeats: extreme phenotype variability? *Am J Med Genet* **64**: 441-444.

270. Knight SJ, Voelckel MA, Hirst MC, Flannery AV, Moncla A, and Davies KE (1994) Triplet repeat expansion at the FRAXE locus and X-linked mild mental handicap. *Am J Hum Genet* **55**: 81-86.
271. Gécz J (2000) *FMR3* is a novel gene associated with *FRAXE* CpG island and transcriptionally silent in *FRAXE* full mutations. *J Med Genet* **37**: 782-784.
272. Robinson MJ, and Cobb MH (1997) Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* **9**: 180-186.
273. Cohen P (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol* **7**: 353-361.
274. Lewis TS, Shapiro PS, and Ahn NG (1998) Signal transduction through MAP kinase cascades. *Adv Cancer Res* **74**: 49-139.
275. English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, and Cobb MH (1999) New insights into the control of MAP kinase pathways. *Exp Cell Res* **253**: 255-270.
276. Marshall CJ (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev* **4**: 82-89.
277. Jones SW, Erikson E, Blenis J, Maller JL, and Erikson RL (1988) A *Xenopus* ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases. *Proc Natl Acad Sci U S A* **85**: 3377-3381.
278. Sturgill TW, Ray LB, Erikson E, and Maller JL (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* **334**: 715-718.
279. Alcorta DA, Crews CM, Sweet LJ, Bankston L, Jones SW, and Erikson RL (1989) Sequence and expression of chicken and mouse rsk: homologs of Xenopus laevis ribosomal S6 kinase. *Mol Cell Biol* **9**: 3850-3859.
280. Zhao Y, Bjorbaek C, Weremowicz S, Morton CC, and Moller DE (1995) RSK3 encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. *Mol Cell Biol* **15**: 4353-4363.
281. Chen RH, Sarnecki C, and Blenis J (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol Cell Biol* **12**: 915-927.
282. Moller DE, Xia CH, Tang W, Zhu AX, and Jakubowski M (1994) Human rsk isoforms: cloning and characterization of tissue-specific expression. *Am J Physiol* **266**: C351-C359.
283. Xing J, Ginty DD, and Greenberg ME (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**: 959-963.
284. Ghoda L, Lin X, and Greene WC (1997) The 90-kDa ribosomal S6 kinase (pp90rsk) phosphorylates the N-terminal regulatory domain of IκBα and stimulates its degradation *in vitro*. *J Biol Chem* **272**: 21281-21288.
285. Schouten GJ, Vertegaal AC, Whiteside ST, Israel A, Toebes M, Dorsman JC, van der Eb AJ, and Zantema A (1997) IkappaB alpha is a target for the mitogen-activated 90 kDa ribosomal S6 kinase. *EMBO J* **16**: 3133-3144.
286. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, and Lannigan DA (1998) $pp90^{rsk1}$ regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* **18**: 1978-1984.
287. Nakajima T, Fukamizu A, Takahashi J, Gage FH, Fisher T, Blenis J, and Montminy MR (1996) The signal-dependent coactivator CBP is a nuclear target for pp90RSK. *Cell* **86**: 465-474.
288. Angenstein F, Greenough WT, and Weiler IJ (1998) Metabotropic glutamate receptor-initiated translocation of protein kinase p90rsk to polyribosomes: a possible factor regulating synaptic protein synthesis. *Proc Natl Acad Sci USA* **95**: 15078-15083.
289. Sutherland C, Leighton IA, and Cohen P (1993) Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem J* **296**: 15-19.
290. Wong EV, Schaefer AW, Landreth G, and Lemmon V (1996) Involvement of p90rsk in neurite outgrowth mediated by the cell adhesion molecule L1. *J Biol Chem* **271**: 18217-18223.
291. Douville E, and Downward J (1997) EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2. *Oncogene* **15**: 373-383.
292. Palmer A, Gavin AC, and Nebreda AR (1998) A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J* **17**: 5037-5047.
293. Sassone-Corsi P, Mizzen CA, Cheung P, Crosio C, Monaco L, Jacquot S, Hanauer A, and Allis CD (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science* **285**: 886-891.
294. Frodin M, Gammeltoft S (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* **151**: 65-77.
295. Trivier E, De Cesare D, Jacquot S, Pannetier S, Zackai E, Young I, Mandel J-L, Sassone-Corsi P, and Hanauer A (1996) Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. *Nature* **384**: 567-570.
296. Merienne K, Jacquot S, Trivier E, Pannetier S, Rossi A, Scott C, Schinzel A, Castellan C, Kress W, and Hanauer A (1998) Rapid immunoblot and kinase assay tests for a syndromal form of X linked mental retardation: Coffin-Lowry syndrome. *J Med Genet* **35**: 890-894.
297. Jacquot S, Merienne K, De Cesare D, Pannetier S, Mandel JL, Sassone-Corsi P, and Hanauer A (1998) Mutation analysis of the RSK2 gene in Coffin-Lowry patients: extensive allelic heterogeneity and a high rate of de novo mutations. *Am J Hum Genet* **63**: 1631-1640.
298. Carew TJ (1996) Molecular enhancement of memory formation. *Neuron* **16**: 5-8.
299. Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, and Bird A (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* **69**: 905-914.
300. Nan X, Meehan RR, and Bird A (1993) Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res* **21**: 4886-4892.
301. Nan X, Ng H-H, Johnson CA, Laherty CD, Turner BM, Eisenman RN, and Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**: 386-389.
302. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, and Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**: 187-191.
303. Hagberg B, Goutieres F, Hanefeld F, Rett A, and Wilson J (1985) Rett syndrome: criteria for inclusion and exclusion. *Brain Dev* **7**: 372-373.
304. Bienvenu T, Carrié A, de Roux N, Vinet MC, Jonveaux P, Couvert P, Villard L, Arzimanoglou A, Beldjord C, Fontes M, Tardieu M, and Chelly J (2000) *MECP2* mutations account for most cases of typical forms of Rett syndrome. *Hum Mol Genet* **9**: 1377-1384.
305. Wan M, Lee SSJ, Zhang X, Houwink-Manville I, Song H-R, Amir RE, Budden S, Naidu S, Pereira JLP, Lo IFM et al (1999) Rett syndrome and beyond: recurrent spontaneous and familial *MECP2* mutations at CpG hotspots. *Am J Hum Genet* **65**: 1520-1529.
306. Van den Veyver IB, and Zoghbi HY (2000) Methyl-CpG-binding protein 2 mutations in Rett syndrome. *Curr Opin Genet Dev* **10**: 275-279.
307. Amir RE, Van den Veyver IB, Schultz R, Malicki DM, Tran CQ, Dahle EJ, Philippi A, Timar L, Percy AK, Motil KJ et al (2000) Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes. *Ann Neurol* **47**: 670-679.
308. Cheadle JP, Gill H, Fleming N, Maynard J, Kerr A, Leonard H, Krawczak M, Cooper DN, Lynch S, Thomas N et al (2000) Long-read sequence analysis of the *MECP2* gene in Rett syndrome patients: correlation of disease severity with mutation type and location. *Hum Mol Genet* **9**: 1119-1129.
309. Hampson K, Woods CG, Latif F, and Webb T (2000) Mutations in the *MECP2* gene in a cohort of girls with Rett syndrome. *J Med Genet* **37**: 610-612.

310. Huppke P, Laccone F, Krämer N, Engel W, and Hanefeld F (2000) Rett syndrome: analysis of *MECP2* and clinical characterization of 31 patients. *Hum Mol Genet* **9**: 1369-1375.
311. Kim S-J, and Cook EH (2000) Novel *de novo* nonsense mutation of *MECP2* in a patient with Rett syndrome. *Hum Mutat* **15** : 382-383.
312. Obata K, Matsuishi T, Yamashita Y, Fukuda T, Kuwajima K, Horiuchi I, Nagamitsu S, Iwanaga R, Kimura A, Omori I et al (2000) Mutation analysis of the methyl-CpG binding protein 2 gene (*MECP2*) in patients with Rett syndrome. *J Med Genet* **37**: 608-610.
313. Xiang F, Buervenich S, Nicolao P, Bailey MES, Zhang Z, and Anvret M (2000) Mutation screening in Rett syndrome patients. *J Med Genet* **37**: 250-255.
314. D'Esposito M, Quaderi NA, Ciccodicola A, Bruni P, Esposito T, D'Urso M, and Brown SD (1996) Isolation, physical mapping, and northern analysis of the X-linked human gene encoding methyl CpG-binding protein, MECP2. *Mamm Genome* **7**: 533-535.
315. Hackam AS (2000) Rett syndrome: The influence of dysregulated gene splicing. *Clin Genet* **57**: 170-171.
316. Villard L, Kpebe A, Cardoso C, Chelly J, Tardieu M, and Fontes M (2000) Two affected boys in a Rett syndrome family: clinical and molecular findings. *Neurology* **55**: 1188-1193.
317. Clayton-Smith J, Watson P, Ramsden S, and Black GCM (2000) Somatic mutation in MECP2 as a non-fatal neurodevelopmental disorder in males. *Lancet* **356**: 830-832.
318. Meloni I, Bruttini M, Longo I, Mari F, Rizzolio F, D'Adamo P, Denvriendt K, Fryns J-P, Toniolo D, and Renieri A (2000) A mutation in the Rett syndrome gene, *MECP2*, causes X-linked mental retardation and progressive spasticity in males. *Am J Hum Genet* **67**: 982-985.
319. Imessaoudene B, Bonnefont J-P, Royer G, Cormier-Daire V, Lyonnet S, Lyon G, Munnich A, and Amiel J (2001) *MECP2* mutation in non-fatal, non-progressive encephalopathy in a male. *J Med Genet* **38**: 171-174.
320. Watson P, Black G, Ramsden S, Barrow M, Super M, Kerr B, and Clayton-Smith J (2001) Angelman syndrome phenotype associated with mutations in *MECP2*, a gene encoding a methyl CpG binding protein. *J Med Genet* **38**: 224-228.
321. Yntema HG, Oudakker AR, Kleefstra T, Hamel BCJ, van Bokhoven H, Chelly J, Kalscheuer VM, Fryns J-P, Raynaud M, Moizard M-P, and Moraine C. In frame deletion in MECP2 causes mild nonspecific mental retardation. *Am J Med Genet* (in press)
322. Dragich J, Houwink-Manville I, and Schanen C (2000) Rett syndrome: a surprising result of mutation in *MECP2*. *Hum Mol Genet* **9**: 2365-2375.
323. Oldfors A, Sourander P, Armstrong DL, Percy AK, Witt-Engerstrom I, and Hagberg BA (1990) Rett syndrome: cerebellar pathology. *Pediatr Neurol* **6**: 310-314.
324. Armstrong D, Dunn JK, Antalffy B, and Trivedi R (1995) Selective dendritic alterations in the cortex of Rett syndrome. *J Neuropathol Exp Neurol* **54**: 195-201.
325. Bauman ML, Kemper TL, and Arin DM (1995) Microscopic observations of the brain in Rett syndrome. *Neuropediatrics* **26**: 105-108.
326. Iguchi-Ariga SM, Schaffner W (1989) CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. *Genes Dev* **3**: 612-619.
327. Holliday R (1999) Is there an epigenetic component in long-term memory? *J Theor Biol* **200**: 339-341.
328. Kingston RE, Bunker CA, and Imbalzano AN (1996) Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev* **10**: 905-920.
329. Kingston RE, Narlikar GJ (1999) ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* **13**: 2339-2352.
330. Quinn J, Fyrberg AM, Ganster RW, Schmidt MC, and Peterson CL (1996) DNA-binding properties of the yeast SWI/SNF complex. *Nature* **379**: 844-847.
331. Cairns BR (1998) Chromatin remodeling machines: similar motors, ulterior motives. *Trends Biochem Sci* **23**: 20-25.
332. Phelan ML, Sif S, Narlikar GJ, and Kingston RE (1999) Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. *Mol Cell* **3**: 247-253.
333. Cairns BR, Erdjument-Bromage H, Tempst P, Winston F, and Kornberg RD (1998) Two actin-related proteins are shared functional components of the chromatin-remodeling complexes RSC and SWI/SNF. *Mol Cell* **2**: 639-651.
334. Zhao K, Wang W, Rando OJ, Xue Y, Swiderek K, Kuo A, and Crabtree GR (1998) Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell* **95**: 625-636.
335. Sudarsanam P, Iyer VR, Brown PO, and Winston F (2000) Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **97**: 3364-3369.
336. Holstege FC, Jennings EG, Wyrick JJ, Lee TI, Hengartner CJ, Green MR, Golub TR, Lander ES, and Young RA (1998) Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**: 717-728.
337. Strahl BD, and Allis CD (2000) The language of covalent histone modifications. *Nature* **403**: 41-45.
338. Wilson CJ, Chao DM, Imbalzano AN, Schnitzler GR, Kingston RE, and Young RA (1996) RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell* **84**: 235-244.
339. Pazin MJ, and Kadonaga JT (1997) SWI2/SNF2 and related proteins: ATP-driven motors that disrupt protein-DNA interactions? *Cell* **88**: 737-740.
340. Elgin SC (1996) Heterochromatin and gene regulation in Drosophila. *Curr Opin Genet Dev* **6**: 193-202.
341. Picketts DJ, Higgs DR, Bachoo S, Blake DJ, Quarrell OWJ, and Gibbons RJ (1996) *ATRX* encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. *Hum Mol Genet* **5**: 1899-1907.
342. Hoeijmakers JHJ (1993) Nucleotide excision repair. II: From yeast to mammals. *Trends Genet* **9**: 211-217.
343. Villard L, Lossi A-M, Cardoso C, Proud V, Chiarioni P, Colleaux L, Schwartz C, and Fontés M (1997) Determination of the genomic structure of the XNP/ATRX gene encoding a potential zinc finger helicase. *Genomics* **43**: 149-155.
344. Cardoso C, Timsit S, Villard L, Khrestchatisky M, Fontes M, and Colleaux L (1998) Specific interaction between the XNP/ATR-X gene product and the SET domain of the human EZH2 protein. *Hum Mol Genet* **7**: 679-684.
345. Rastelli L, Chan CS, and Pirrotta V (1993) Related chromosome binding sites for zeste, suppressors of zeste and Polycomb group proteins in Drosophila and their dependence on Enhancer of zeste function. *EMBO J* **12**: 1513-1522.
346. Le Douarin B, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, and Chambon P (1996) A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. *EMBO J* **15**: 6701-6715.
347. Bérubé NG, Smeenk CA, and Picketts DJ (2000) Cell cycle-dependent phosphorylation of the ATRX protein correlates with changes in nuclear matrix and chromatin association. *Hum Mol Genet* **9**: 539-547.
348. McDowell TL, Gibbons RJ, Sutherland H, O'Rourke DM, Bickmore WA, Pombo A, Turley H, Gatter K, Picketts DJ, Buckle VJ et al (1999) Localization of a putative transcriptional regulator (ATRX) at pericentromeric heterochromatin and the short arms of acrocentric chromosomes. *Proc Natl Acad Sci USA* **96**: 13983-13988.
349. Cardoso C, Lutz Y, Mignon C, Compe E, Depetris D, Mattei M-G, Fontes M, and Colleaux L (2000) *ATR-X* mutations cause impaired nuclear location and altered DNA binding properties of the XNP/ATR-X protein. *J Med Genet* **37**: 746-751.
350. Antequera F, and Bird A (1993) Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci USA* **90**: 11995-11999.
351. Tazi J, and Bird A (1990) Alternative chromatin structure at CpG islands. *Cell* **60**: 909-920.

352. Tse C, Sera T, Wolffe AP, and Hansen JC (1998) Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. *Mol Cell Biol* **18**: 4629-4638.
353. Schnitzler G, Sif S, and Kingston RE (1998) Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. *Cell* **94**: 17-27.
354. Lorch Y, Cairns BR, Zhang M, and Kornberg RD (1998) Activated RSC-nucleosome complex and persistently altered form of the nucleosome. *Cell* **94**: 29-34.
355. Flint J, Tufarelli C, Peden J, Clark K, Daniels RJ, Hardison R, Miller W, Philipsen S, Tan-Un KC, McMorrow T et al (2001) Comparative genome analysis delimits a chromosomal domain and identifies key regulatory elements in the α globin cluster. *Hum Mol Genet* **10**: 371-382.
356. Gibbons RJ, McDowell TL, Raman S, O'Rourke DM, Garrick D, Ayyub H, and Higgs DR (2000) Mutations in *ATRX*, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat Genet* **24**: 368-371.
357. Robertson KD, and Wolffe AP (2000) DNA methylation in healt and disease. *Nature Rev (genetics)* **1**: 11-19.
358. Goto K, Numata M, Komura JI, Ono T, Bestor TH, and Kondo H (1994) Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* **56**: 39-44.
359. Bannister AJ, and Kouzarides T (1996) The CBP co-activator is a histone acetyltransferase. *Nature* **384**: 641-643.
360. Renshaw MW, Toksoz D, and Schwartz MA (1996) Involvement of the small GTPase rho in integrin-mediated activation of mitogen-activated protein kinase. *J Biol Chem* **271**: 21691-21694.
361. Turner G, and Partington M (2000) Recurrence risks in undiagnosed mental retardation. *J Med Genet* **37**: E45.
362. Turner G, and Partington MW (1991) Genes for intelligence on the X chromosome. *J Med Genet* **28**: 429.
363. Turner G (1996) Intelligence and the X chromosome. *Lancet* **347**: 1814-1815.
364. Lubs HA (1999) The other side of the coin: a hypothesis concerning the importance of genes for high intelligence and evolution of the X chromosome. *Am J Med Genet* **85**: 206-208.
365. Morton NE (1992) Genes for intelligence on the X chromosome. *J Med Genet* **29**: 71.
366. Hook EB (1996) Intelligence and the X chromosome. *Lancet* **348**: 826.
367. Hedges LV, and Nowell A (1995) Sex differences in mental test scores, variability, and numbers of high-scoring individuals. *Science* **269**: 41-45.
368. Knight SJ, Regan R, Nicod A, Horsley SW, Kearney L, Homfray T, Winter RM, Bolton P, and Flint J (1999) Subtle chromosomal rearrangements in children with unexplained mental retardation. *Lancet* **354**: 1676-1681.
369. Slavotinek A, Rosenberg M, Knight S, Gaunt L, Fergusson W, Killoran C, Clayton-Smith J, Kingston H, Campbell RH, Flint J, Donnai D, and Biesecker L (1999) Screening for submicroscopic chromosome rearrangements in children with idiopathic mental retardation using microsatellite markers for the chromosome telomeres. *J Med Genet* **36**: 405-411.
370. Gécz J, and Mulley J (2000) Genes for cognitive function: developments on the X. *Genome Res* **10**: 157-163.
371. Higgins JJ, Rosen DR, Loveless JM, Clyman JC, and Grau MJ (2000) A gene for nonsyndromic mental retardation maps to chromosome 3p25-pter. *Neurology* **55**: 335-340.
372. Sudbrak R, Wieczorek G, Nuber UA, Mann W, Kirchner R, Erdogan F, Brown CJ, Wöhrle D, Sterk P, Kalscheuer VM et al (2001) X chromosome-specific cDNA arrays: identification of genes that escape from X-inactivation and other applications. *Hum Mol Genet* **10**: 77-83.
373. Strauss E (2001) AAAS meeting. Stem cells make brain cells. *Science* **291**: 1689-1690.
374. Vogel G (2000) Stem cells: new excitement, persistent questions. *Science* **290**: 1672-1674.
375. Isacson O, Costantini L, Schumacher JM, Cicchetti F, Chung S, and Kim K-S (2001) Cell implantation therapies for Parkinson's disease using neural stem, transgenic or xenogeneic donor cells. *Parkinsonism Relat Disord* **7**: 205-212.
376. de Vries BBA, van den Ouweland AMW, Mohkamsing S, Duivenvoorden HJ, Mol E, Gelsema K, van Rijn M, Halley DJJ, Sandkuijl LA, Oostra BA et al (1997) Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey. *Am J Hum Genet* **61**: 660-667.

Appendix 1

Localization of a gene for nonspecific X-linked mental retardation (MRX46) to Xq25-q26

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Published in: *J Med Genet* **35**: 801-805 (1998)

ABSTRACT

We report linkage data on a new large family with nonspecific X-linked mental retardation (MRX), using 24 polymorphic markers covering the entire X-chromosome. We could assign the underlying disease gene, denoted MRX46, to the Xq25-q26 region. MRX46 is tightly linked to the markers DXS8072, HPRT, and DXS294 with a maximum lod score of 5.12 at θ=0. Recombination events were observed with DXS425 in Xq25 and DXS984 at the Xq26-Xq27 boundary, which localizes MRX46 to a 20.9 cM (12 Mb) interval. Several X-linked mental retardation syndromes have been mapped to the same region of the X-chromosome. In addition, the localization of two MRX genes, MRX27 and MRX35, partially overlaps with the linkage interval obtained for MRX46. Although an extension of the linkage analysis for MRX35 revealed only a minimal overlap with MRX46, it cannot be excluded that the same gene is involved in several of these MRX disorders. On the other hand, given the considerable genetic heterogeneity in MRX, one should be extremely cautious in using interfamilial linkage data to narrow down the localization of MRX genes. Therefore, unless the underlying gene(s) is characterized by the analysis of candidate genes, MRX46 can be considered a new independent MRX locus.

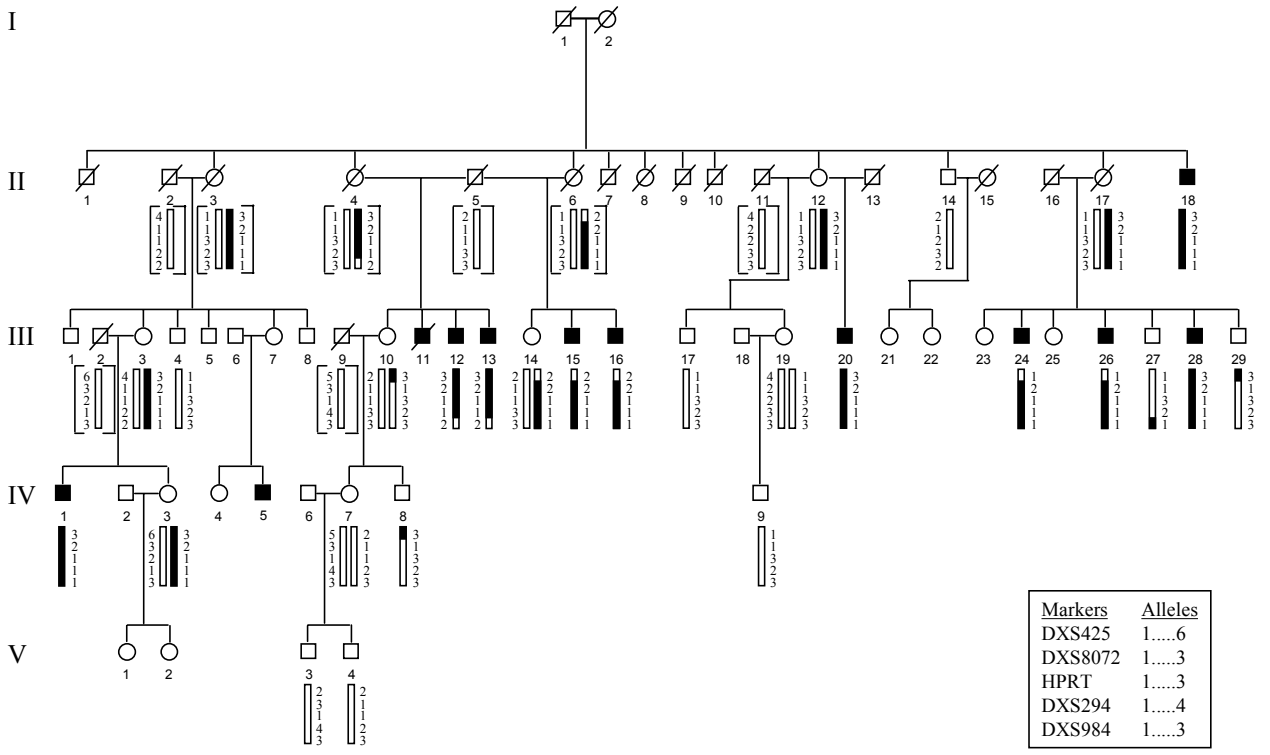

Figure 1. *Five generation family with MRX. Twenty-seven family members, including 10 affected males, were available for DNA study. Haplotypes of the linked markers in Xq25-q26 and the recombined markers delimiting the probable gene location are shown. Deduced haplotypes from subjects who had died are shown between brackets. The disease chromosome is indicated by a black bar.*

INTRODUCTION

X-linked mental retardation (XLMR) is considered to be the most frequent type of mental handicap in males. It has been estimated that mutations in X-chromosomal genes account for 25 to 50 % of all cases of mental retardation[1]. A small part of XLMR can be attributed to recognizable syndromes and to date more than 100 XLMR syndromes (MRXS) have been described[2]. More often, however, the mental handicap is not associated with consistent phenotypic characteristics. This is referred to as nonspecific XLMR or MRX.

Linkage analyses in individual families with MRX have currently shown over 50 loci on the X chromosome which are clustered in eight non-overlapping regions[3,4]. The inclusion of the FRAXE mental retardation gene[5] and the *RAB-GDI* gene[6] suggests a minimum of 10 X linked genes which are involved in non-specific mental retardation. In this report, we present a large family with non-specific X linked mental retardation and the mapping of the underlying gene to Xq25-q26, a region that has rarely been implicated in MRX.

PATIENTS AND METHODS

Clinical report

The family (Figure 1) was ascertained when IV.7 was referred for genetic counseling. The pedigree included 12 mentally retarded males in three generations, nine of whom were clinically examined. In order to determine the mental status of these patients, the following intelligence tests, standardized for school aged children, were used: (1) the Wechsler Intelligence Scale for Children Revised (WISC-R)[7,8], (2) the Coloured Progressive Matrices (CPM)[9,10], (3) the Revised Amsterdam Child Intelligence Test (RAKIT)[11], and (4) McCarthy Scales of Children's Abilities[12]. The RAKIT and McCarthy Scales were applied to assess intellectual functioning below an equivalent of 6 years[13]. Scores of all tests were transferred into age equivalents according to the classification of the American Association of Mental Deficiency (AAMD)[14]. Cytogenetic analysis and molecular study of the *FMR1* gene was performed in several patients. In III.28 the diagnostic tests included cerebral CT scan and metabolic screening. Informed consent was obtained in all instances.

DNA analysis

DNA from 26 relatives was isolated from peripheral blood lymphocytes, according to the procedure by Miller *et al.*[15]. In order to determine the most likely location of the gene, linkage analysis was performed with highly polymorphic markers distributed along the X-chromosome. Analysis of these markers involved amplification by polymerase chain reaction (PCR) carried out in a 96 well Thermal Cycler (MJ Research Inc, Waterston, MA). Each reaction contained 100 ng genomic DNA, 30 ng of each of the primers, in 15 µl 1 ×

Super*taq* buffer (50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.01% (w/v) gelatin) in the presence of ^{32}P-dCTP with 0.06 U Super*taq* (HT Biotechnology Ltd, England). Amplification was achieved by 35 cycles of one minute at 94°C, two minutes at 55°C, and three minutes at 72°C with the locus specific primer pairs registered in the Genome Database (http://www.gdb.org/). The radiolabeled PCR products were mixed with 15 µl sample buffer (95% formamide, 20 mmol/l EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) heated to 95°C for two minutes, and 4 µl of this mixture was separated on a 6.6% denaturing polyacrylamide gel. Subsequently, the gel was dried and exposed overnight to Kodak X-OMAT film to visualize the separated allelic bands.

Two point linkage analyses of the 24 polymorphic markers and the disease locus were performed with the MLINK option of the computer program LINKAGE (version 5.03)[16-18] on the basis of X linked recessive inheritance with full penetrance. The relative order of marker loci was obtained from the Genome Database and the Report of the Sixth International Workshop on X Chromosome Mapping[19].

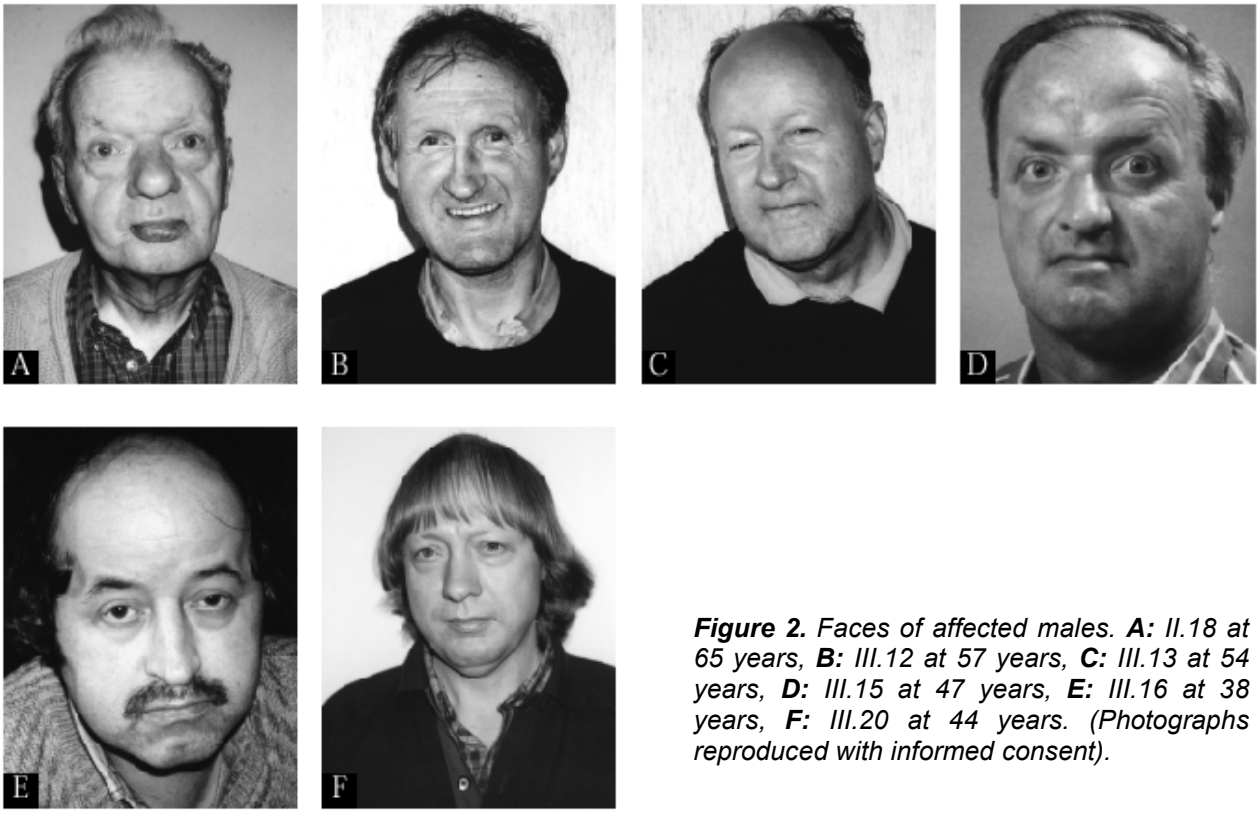

Figure 2. *Faces of affected males.* ***A:*** *II.18 at 65 years,* ***B:*** *III.12 at 57 years,* ***C:*** *III.13 at 54 years,* ***D:*** *III.15 at 47 years,* ***E:*** *III.16 at 38 years,* ***F:*** *III.20 at 44 years. (Photographs reproduced with informed consent).*

RESULTS

Clinical report

In all patients, pregnancy and delivery were uneventful. The mental retardation was noticed during early childhood and appeared non-progressive. None of them had convulsions. All patients were either institutionalized or living and working in a sheltered

environment. Physically they were all healthy. Their behavior was normal, except for the brothers III.24, III.26, and III.28 who were described as difficult to control, chaotic, and autistic-like, and III.16 who showed obsessive behavior. III.11 died at the age of 11 during heart surgery. He was attending a special education unit. Obligate carriers were all normal. Results of the clinical measurements and psychometric studies are summarized in Tables 1 and 2, respectively.

Only minor anomalies were noted in some of the affected males. II.18 (Figure 2A) was seen at the age of 65 years. He was short statured. Hypertelorism, a fleshy nose, full lower lip, kypholordosis, and lower leg varices were noticed. III.12 (Figure 2B) showed no abnormalities except for lower leg varices when seen at the age of 57 years. III.13 (Figure 2C) was examined at the age of 54 years. Except for a left sided hydrocele no abnormalities were detected. III.15 (Figure 2D) and III.16 (Figure 2E) showed no abnormalities on examination at the ages of 47 and 38, respectively. III.20 (Figure 2F) was examined at the age of 44 years. Left sided hydrocele and a large hyperpigmented spot on the back were seen. III.24 showed a highly arched palate, short and broad neck, and flat feet on examination at the age of 34 years. He was short statured. Ophthalmologic examination of III.26 at the age of 29 years showed hypertelorism, myopia gravis ODS, and some lenticular opacities. In III.28 hypertelorism and myopia gravis OD, resulting in amblyopia and divergent strabismus, were seen on ophthalmologic examination. The degree of mental retardation in the affected family members tested varied from mild to profound. It is concluded that pedigree structure and clinical data are fully compatible with a diagnosis of nonspecific X-linked mental retardation.

Table 1. *Summary of clinical measurements (centiles)*

	Height (cm)	Weight (kg)	OFC (cm)	Ear length (mm)	OCD (mm)	ICD (mm)	THL (cm)	Testicular volume (cm^3)
II-18	160 (<3)	56 (<10)	56 (50)	75 (97)	95 (75-97)	38 (>97)	18 (50-75)	20 (50)
III-12	182 (50)	85 (90)	56 (50)	71 (75-97)	81 (3-25)	29 (25-50)	19,5 (97)	22 (50-90)
III-13	168 (<10)	83 (90)	56,5 (50-90)	60 (50)	83 (25)	31 (50)	19 (75-97)	23 (50-90)
III-15	182 (50)	92 (>90)	58,5 (>90)	65 (75)	95 (75-97)	35 (75-97)	18 (50-75)	25 (50-90)
III-16	182 (50)	-	59 (>90)	61 (50)	84 (25-50)	31 (50)	19 (75-97)	-
III-20	172 (3-10)	76 (50-90)	58 (90)	60 (50)	91 (50-75)	32 (50-75)	19 (75-97)	20 (50)
III-24	163 (<3)	75,5 (50-90)	-	-	-	-	-	-
III-26	170 (3-10)	60 (10)	54 (10-50)	60 (50)	105 (>97)	35 (75-97)	19 (75-97)	-
III-28	181 (10-50)	91 (>90)	58 (50-90)	65 (75)	105 (>97)	38 (>97)	19,5 (97)	-

OCD = outer canthal distance
ICD = inner canthal distance
THL = total hand length
– = no data

DNA analysis

For the initial analyses, 23 markers spread along the X chromosome were genotyped on 12 potentially informative family members. The family was informative for all tested markers. Once tentative linkage with HPRT and DXS294 was obtained, another marker in the region, DXS8072, was typed and more affected and unaffected family members were genotyped for the linked markers (26 relatives in total). Table 3 presents the results of the

two point linkage analysis, with the lod scores for the MRX locus and each marker locus. The proximal locus DXS425 at Xq25 and the more distal locus DXS984 at the Xq26-q27 boundary recombined with the disease in the family, delimiting the probable gene location. Significant lod scores were obtained with markers DXS8072, HPRT, and DXS294, indicating that the genetic defect responsible for the disease in this family is located in Xq25-q26, with a maximum lod score of 5.12 at θ=0 (at HPRT). A reconstruction of the haplotypes of the markers in the Xq25-q26 region is shown in Figure 1.

***Table 2**. Summary of psychometric data*

Cases	Intelligence*	Non-verbal reasoning*	Visual/ motor skills*	Adaptive functioning*	Academic achievement		
					Reading†	Writing†	Arithmetic†
II-18	–	+/–	+/–	+/–	+	+	–
III-12	–	–	–	+	+	+	+
III-13	–	+/–	+	–	–	–	–
III-15	–	+/–	+/–	+/–	–	–	+/–
III-16	+/–	+	–	+	+	+/–	+
III-20	–	+/–	‡	+/–	+	+	‡
III-24	–	–	–	–	–	–	–
III-26	– – –	unable	unable	– – –	–	–	–
III-28	– –	unable	– –	– –	–	–	–

*levels of mental and adaptive functioning, transferred into age equivalents:
+ = > 10–9 years (= borderline and above; IQ-range ± 70–84 and above);
+/–= 8–3 and 10–9 years (= mild; IQ range 50/55–70);
– = 5–7 to 8–2 years (= moderate; IQ range 35/40–50/55);
– –= 3–2 to 5–6 years (= severe; IQ range 20/25–35/40);
– – – = < 3–2 years (= profound; IQ range < 20/25).
† levels of academic functioning, transferred into didactic age equivalents (dae):
+ = > 30 months (= adequate);
+/–= 1–29 months (= inadequate);
– = 0 months (= unable).
‡ =no actual information available.

DISCUSSION

We have presented a new family with recessive non-specific X linked mental retardation and assigned the causative genetic defect, MRX46, to Xq25-q26. This region is estimated to comprise a genetic distance of 20.9 cM[20] and a 12 Mb region on the physical map[19].

Seven XLMR syndromes have been assigned to X chromosomal segments overlapping the linkage interval for MRX46 (Figure 3). Huang *et al.*[21] reported on a family with an X-linked mental retardation disorder (MRXS5), including Dandy-Walker malformation, basal ganglia disease and seizures. Börjeson-Forssman-Lehmann syndrome (BFLS)[20,22,23] is characterized by severe mental retardation, microcephaly, hypogonadism, obesity, short stature, and distinct facial anomalies including large ears, prominence of the supraorbital ridge, and ptosis. Malmgren *et al.*[24] reported a family with severe X-linked mental retardation syndrome, linked to DXS294 at Xq26. This so-called Gustavson syndrome includes microcephaly, severe mental retardation, optic atrophy with decreased

vision or blindness, severe hearing defect, characteristic facial features, spasticity, seizures, and restricted joint motility. The patients die during infancy or early childhood[25]. Cowchock syndrome, an X-linked motor-sensory neuropathy type II with deafness and mental retardation has been mapped between DXS425 and HPRT[26,27]. Recently, some of us described a family presenting with varying degrees of mental retardation in conjunction with isolated growth hormone deficiency[28]. The underlying genetic defect for this disorder could be assigned to the Xq24-q27.3 region. Interestingly, in a recent report, an unrelated family with similar symptoms is described in which the genetic defect is localized to the same region[29]. In this family, X-linked recessive panhypopituitarism (XPH) is associated with a duplication of the DXS102 locus, suggesting that this phenotype is caused by a gene dosage effect. Lesch-Nyhan syndrome (OMIM 308000) is another well known syndrome with MR, caused by mutations in the *HPRT1* gene. It cannot be ruled out that one of the genes involved in these MR syndromes is also causative in MRX46, but given the pronounced distinctive features of each syndrome, it is difficult to imagine how mutations in one such gene may cause (severe) non-specific MR.

Table 3. *Lod scores between MRX46 and markers along the X chromosome (in order from Xpter to Xqter)*

	θ 0.000	0.050	0.100	0.200	0.300	0.400
DXS1060	– ∞	–1.93	–0.91	–0.08	0.19	0.19
GHGxG	– ∞	–2.05	–1.01	–0.16	0.13	0.17
DXS443	– ∞	–1.96	–1.14	–0.42	–0.11	0.01
DMD	– ∞	–6.04	–4.01	–2.07	–1.03	–0.39
DXS538	– ∞	0.02	0.40	0.52	–0.35	0.10
DXS7	– ∞	–0.68	–0.29	–0.15	–0.26	–0.29
DXS1003	– ∞	–6.34	–4.30	–2.34	–1.26	–0.53
ALAS2	– ∞	–2.23	–1.19	–0.35	–0.04	0.02
DXS453	– ∞	–5.46	–3.72	–2.05	–1.12	–0.49
DXS559	– ∞	–2.07	–1.34	–0.77	–0.49	–0.23
DXS986	– ∞	–2.07	–1.34	–0.77	–0.49	–0.23
DXS3	– ∞	–0.79	–0.39	–0.23	–0.30	–0.28
DXS178	– ∞	–2.07	–1.34	–0.77	–0.49	–0.23
Col4A5	– ∞	–2.07	–1.34	–0.77	–0.48	–0.23
DXS424	– ∞	–1.96	–1.14	–0.42	–0.11	0.01
DXS1001	– ∞	–0.96	–0.25	0.21	0.27	0.15
DXS425	– ∞	0.14	0.96	1.31	1.09	0.57
DXS8072	4.43	3.99	3.54	2.60	1.63	0.66
HPRT	5.12	4.70	4.26	3.32	2.28	1.12
DXS294	4.95	4.51	4.04	3.07	2.03	0.96
DXS984	– ∞	2.14	2.34	2.10	1.53	0.81
FraXAc2	– ∞	0.09	0.28	0.35	0.28	0.16
DXS1113	– ∞	–0.06	0.15	0.25	0.22	0.13
DXS1108	– ∞	–0.06	0.15	0.25	0.22	0.13

Very recently, two novel MRX loci encompassing Xq26, MRX27, and MRX35 have been uncovered by linkage analysis in apparently unrelated families from the United Kingdom and Belgium, respectively[30-32]. MRX27 has been localized to the Xq24-q26 region with flanking recombination events in affected males at DXS424 and DXS102, which spans all but the distal portion of the MRX46 linkage interval (Figure 3)[31]. MRX35 maps to Xq22-q26 with flanking markers DXS178 and HPRT[32]. DNA samples from this family (kindly provided by Professor JP Fryns and Dr P Raeymaekers) allowed us to assign DXS8072 as the distal flanking marker in MRX35. Hence, the overlap between MRX46 and MRX35

comprises a region of less than 2 cM at the Xq25-q26 boundary (Figure 3). If the same gene is involved in the three MRX families that map to Xq26, the region to search for candidate genes would be significantly reduced. However, given the genetic heterogeneity of MRX one should be extremely cautious of using interfamilial linkage data to narrow down the localization of MRX genes. This is especially true since MRX46 seems to differ from MRX27 and MRX35 in that the variability and severity of the mental handicap is more pronounced and, yet, carrier females never exhibit any mental impairment. Therefore, the whole Xq26 band has to be considered for identification of candidate genes in the family presented here. According to the physical map of Pilia *et al.*[33] five genes for which the complete coding region is known are located in this region (Figure 3). In addition, the human transcript map[34] (http://www.ncbi.nlm.nih.gov/SCIENCE96/) reports more than 50 expressed sequence tags for this region. Unfortunately, expression data for the corresponding genes are not available and it is therefore not possible at this stage sensibly to select likely candidate genes for MR from this gene pool.

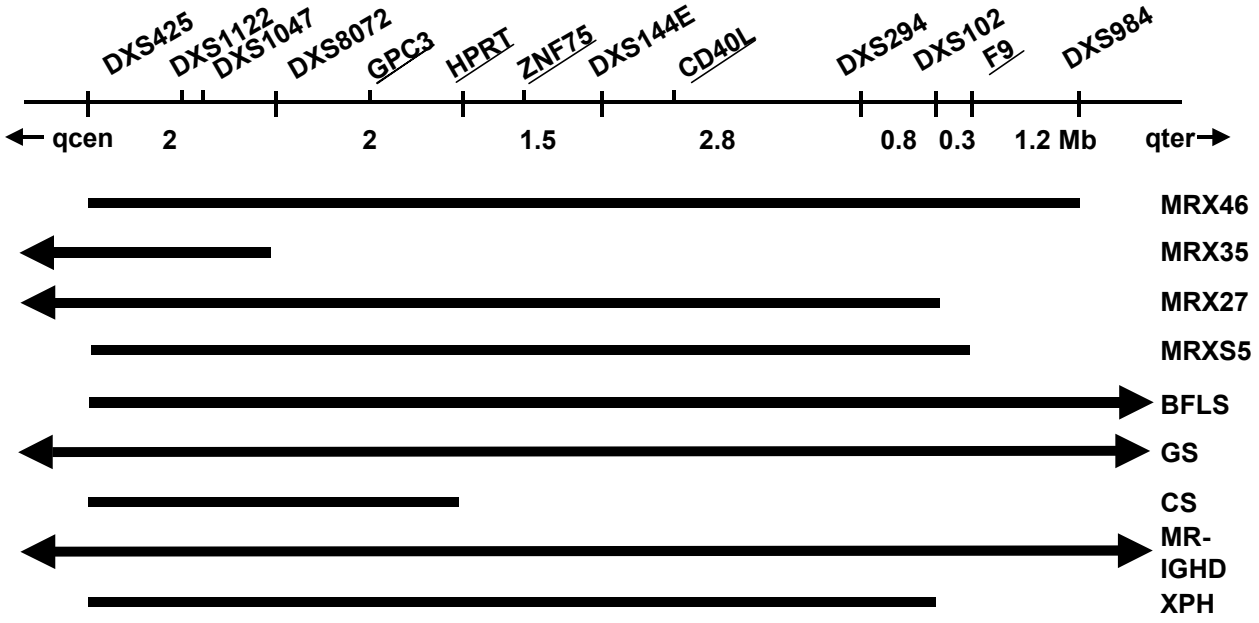

Figure 3. *Localization of XLMR conditions in the Xq25-q26 region. The top bar shows the order of relevant markers and genes (underlined) on a Mb scale. The horizontal bars indicate the linkage intervals. Arrowheads at the end of the bars indicate that the linkage interval extends beyond the chromosomal region shown. BFLS: Börjeson-Forsmann-Lehman syndrome; GS: Gustavson syndrome; CS: Cowchock syndrome; MR-IGHD: mental retardation with isolated growth hormone deficiency; XPH: X-linked recessive panhypopituitarism.*

REFERENCES

1. Kerr B, Turner G, Mulley J, Gedeon A, and Partington M (1991) Non-specific X-linked mental retardation. *J Med Genet* **28**: 378-382.
2. Lubs HA, Chiurazzi P, Arena JF, Schwartz C, Tranebjaerg L, and Neri G (1996) XLMR genes: update 1996. *Am J Med Genet* **64**: 147-157
3. Lubs H, Chiurazzi P, Arena J, Schwartz C, Tranebjaerg L, and Neri G (1999) XLMR genes: update 1998. *Am J Med Genet* **83**: 237-247.
4. Gedeon AK, Donnelly AJ, Mulley JC, Kerr B, and Turner G (1996) How many X-linked genes for non-specific mental retardation (MRX) are there? *Am J Med Genet* **64**: 158-162.
5. Gecz J, Gedeon AK, Sutherland GR, and Mulley JC (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation. *Nat Genet* **13**: 105-108.
6. D'Adamo P, Menegon A, Lo Nigro C, Grasso M, Gulisano M, Tamanini F, Bienvenu T, Gedeon AK, Oostra B, Wu S-K, Tandon A, Valtorta F, Balch WE, Chelly J, and Toniolo D (1998) Mutations in *GDI1* are responsible for X-linked non-specific mental retardation. *Nat Genet* **19-6**: 134-139.
7. Wechsler D (1974) Wechsler intelligence scale for children-revised. New York: Psychological Corporation.
8. Van der Steene G, van Haasen PP, de Bruyn E.E.J., Coetsier P, Pijl YJ, Poortinga YH, Spelberg HC, Spoelders R, and Stinissen R (1986) Intelligence scale for children-revised. Dutch Version. Lisse: Swets & Zeitlinger.
9. Raven JC (1965) Guide to using the coloured progressive matrices. London: Lewis.
10. Van Bon WHJ (1986) Raven's coloured progressive matrices; Nederlandse normen en enige andere uitkomsten van onderzoek. Lisse: Swets & Zeitlinger.
11. Bleichrodt N, Drenth PJD, Zaal J.M., and Resing WCM (1984) Revisie Amsterdamse kinder intellligentietest; instructie, normen, psychometrische gegevens. Lisse: Swets % Zeitlinger.
12. Van der Meulen BF and Smrkovsky M (1985) McCarthy ontwikkelingsschalen. Lisse: Swets & Zeitlinger.
13. Bleichrodt N, Resing WCM, Drenth PJD, and Zaal J.M. (1987) Intelligentiemeting bij kinderen. Lisse: Swets & Zeitlinger.
14. Grossman HJ (1977) Manual on terminology and classification in mental retardation. Washington DC: AAMD.
15. Miller SA, Dykes DD, and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**: 1215-1215.
16. Lathrop GM, Lalouel JM, Julier C, and Ott J (1984) Strategies for multilocus linkage analysis in humans. *Proc Natl Acad Sci USA* **81**: 3443-3446.
17. Lathrop GM and Lalouel JM (1984) Easy calculations of lod scores and genetic risks on small computers. *Am J Hum Genet* **36**: 460-465.
18. Lathrop GM, Lalouel JM, and White RL (1986) Construction of human linkage maps: likelihood calculations for multilocus linkage analysis. *Genet Epidemiol* **3**: 39-52.
19. Nelson DL, Ballabio A, Cremers F, Monaco AP, and Schlessinger D (1995) Report of the sixth international workshop on X chromosome mapping 1995. *Cytogenet Cell Genet* **71**: 307-342.
20. Gedeon AK, Kozman HM, Robinson H, Pilia G, Schlessinger D, Turner G, and Mulley JC (1996) Refinement of the background genetic map of Xq26-q27 and gene localisation for Borjeson-Forssman-Lehmann Syndrome. *Am J Med Genet* **64**: 63-68.
21. Huang THM, Hejtmancik JF, Edwards A, Pettigrew AL, Herrera CA, Hammond HA, Caskey CT, Zoghbi HY, and Ledbetter DH (1991) Linkage of the gene for an X-linked mental retardation disorder to a hypervariable (AGAT)n repeat motif within the human hypoxanthine phosphoribosyltransferase (HPRT) locus (Xq26). *Am J Hum Genet* **49**: 1312-1319.
22. Turner G, Gedeon A, Mulley J, Sutherland G, Rae J, Power K, and Arthur I (1989) Börjeson-Forssman-Lehmann syndrome: Clinical manifestations and gene localization to Xq26-27. *Am J Med Genet* **34**: 463-469.
23. Mathews KD, Ardinger HH, Nishimura DY, Buetow KH, Murray JC, and Bartley JA (1989) Linkage localization of Börjeson-Forssman-Lehmann syndrome. *Am J Med Genet* **34**: 470-474.
24. Malmgren H, Sundvall M, Dahl N, Gustavson K-H, Annerén G, Wadelius C, Steén-Bondeson M-L, and Pettersson U (1993) Linkage mapping of a severe X-linked mental retardation syndrome. *Am J Hum Genet* **52**: 1046-1052.
25. Gustavson K-H, Annerén G, Malmgren H, Dahl N, Ljunggren C-G, and Bäckman H (1993) New X-linked syndrome with severe mental retardation, severely impaired vision, severe hearing defect, epileptic seizures, spasticity, restricted joint mobility, and early death. *Am J Med Genet* **45**: 654-658.
26. Cowchock FS, Duckett SW, Streletz LJ, Graziani LJ, and Jackson LG (1985) X-linked motor-sensory neuropathy type-II with deafness and mental retardation: A new disorder. *Am J Med Genet* **20**: 307-315.
27. Priest JM, Fischbeck KH, Nouri N, and Keats BJB (1995) A locus for axonal motor-sensory neuropathy with deafness and mental retardation maps to Xq24-26. *Genomics* **29** : 409-412.
28. Hamel BCJ, Smits APT, Otten BJ, van den Helm B, Ropers H-H, and Mariman ECM (1996) Familial X-linked mental retardation and isolated growth hormone deficiency: Clinical and molecular findings. *Am J Med Genet* **64**: 35-41.
29. Lagerström-Fermér M, Sundvall M, Johnsen E, Warne GL, Forrest SM, Zajac JD, Rickards A, Ravine D, Landegren U, and Pettersson U (1997) X-linked recessive panhypopituitarism associated with a regional duplication in Xq25-q26. *Am J Hum Genet* **60**: 910-916.
30. Glass IA, White EM, Pope MJ, Pirrit LA, Cockburn F, and Connor JM (1991) Linkage analysis in a large family with nonspecific X-linked mental retardation. *Am J Med Genet* **38**: 240-243.
31. Gedeon AK, Glass IA, Connor JM, and Mulley JC (1996) Genetic localisation of MRX27 to Xq24-26 defines another discrete gene for non-specific X-linked mental retardation. *Am J Med Genet* **64**: 121-124.
32. Gu XX, Decorte R, Marynen P, Fryns J-P, Cassiman J-J, and Raeymaekers P (1996) Localisation of a new gene for non-specific mental retardation to Xq22-q26 (MRX35). *J Med Genet* **33**: 52-55.
33. Pilia G, Macmillan S, Nagaraja R, Mumm S, Weissenbach J, and Schlessinger D (1996) YAC/STS map of 9 Mb of Xq26 at 100-kb resolution, localizing 6 ESTs, 6 genes, and 32 genetic markers. *Genomics* 34: 55-62.
34. Schuler GD, Boguski MS, Stewart EA, Stein LD, Gyapay G, Rice K, White RE, Rodriguez-Tome P, Aggarwal A, Bajorek E, Bentolila S, Birren BB, Butler A, Castle AB, Chiannilkulchai N, Chu A, Clee C, Cowles S, Day PJ, Dibling T, Drouot N, Dunham I, Duprat S, East C, and Hudson TJ (1996) A gene map of the human genome. *Science* 274: 540-546.

Appendix 2

X-linked mental retardation: Evidence for a recent mutation in a five-generation family (MRX65) linked to the pericentromeric region

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Published in: *Am J Med Genet* **85**: 305-308 (1999)

ABSTRACT

We report linkage analysis in a new family with nonspecific X-linked mental retardation, using 27 polymorphic markers covering the entire X-chromosome. We could assign the underlying disease gene, denoted MRX65, to the pericentromeric region, with flanking markers DXS573 in Xp11.3 and DXS990 in Xq21.33. A maximum LOD score of 3.64 was found at markers ALAS2 (Xp11.22) and DXS453 (Xq12) at θ=0. Twenty-five of the 58 reported MRX families are linked to a region that is partially overlapping with the region reported here. Extension of the pedigree showed a number of unaffected distant relatives with haplotypes corresponding to the disease locus. Apparently, a new mutation in a female is causative for the disease in the family reported here. Furthermore, we show the importance of the combining clinical, cytogenetic, and molecular studies since one of the family members, expected to be affected by the same genetic defect, has a 48,XXXY karyotype.

INTRODUCTION

X-linked mental retardation (XLMR) is clinically variable and genetically heterogeneous. Fifty-eight families with nonspecific XLMR (MRX) are listed in a review by Lubs *et al*.[1]. Since only three genes for MRX have been identified, *FMR2*[2], *GDI1*[3] and *oligophrenin-1*[4], the vast majority of genes are not known. Twenty-five of the 58 reported families show linkage to the Xp11-q21 region[1]. The large number of families mapping to this region suggests either a clustering of MRX genes or the existence of one or a few genes that are frequently mutated. We report yet another family with nonspecific XLMR (MRX65) that maps to the pericentromeric region. Haplotype analysis shows that a de novo mutation in one of the females is causative for the condition in this family.

PATIENTS AND METHODS

Clinical report

The family (Figure 1) was ascertained when IV-15 was referred for genetic counseling. Initially a branch of the family with six affected males in three generations was tested (Figure 1A). In order to determine the mental status of the patients suspected for mental retardation, we employed the three criteria form the DSM-IV definition of mental retardation[5]: 1) intelligence: IQ of 70 or lower; 2) significant limitations in adaptive functioning; 3) onset before the age of 18 years. In order to be able to establish a qualitative description of intellectual functioning, intelligence tests, standardized for school-aged children, were used: the Wechsler Intelligence Scale for Children, Revised (WISC-R)[6,7] and the Revised Amsterdam Child Intelligence Test (RAKIT)[8]. Initially, all patients suspected for mental retardation were assessed with subtests of the WISC-R. If these proved to be too

difficult and a floor effect threatened, we exchanged them for the RAKIT. The second criterion was measured with a Dutch questionnaire[9,10]. The age of onset was obtained by anamnestic information. Scores were translated into age equivalents according to the classification of the American Association of Mental Deficiency (AAMD)[11].

Once tentative linkage was established, genealogical research lead to an extended pedigree with two additional affected males (III-5 and IV-22), one of whom had already died (Figure 1B).

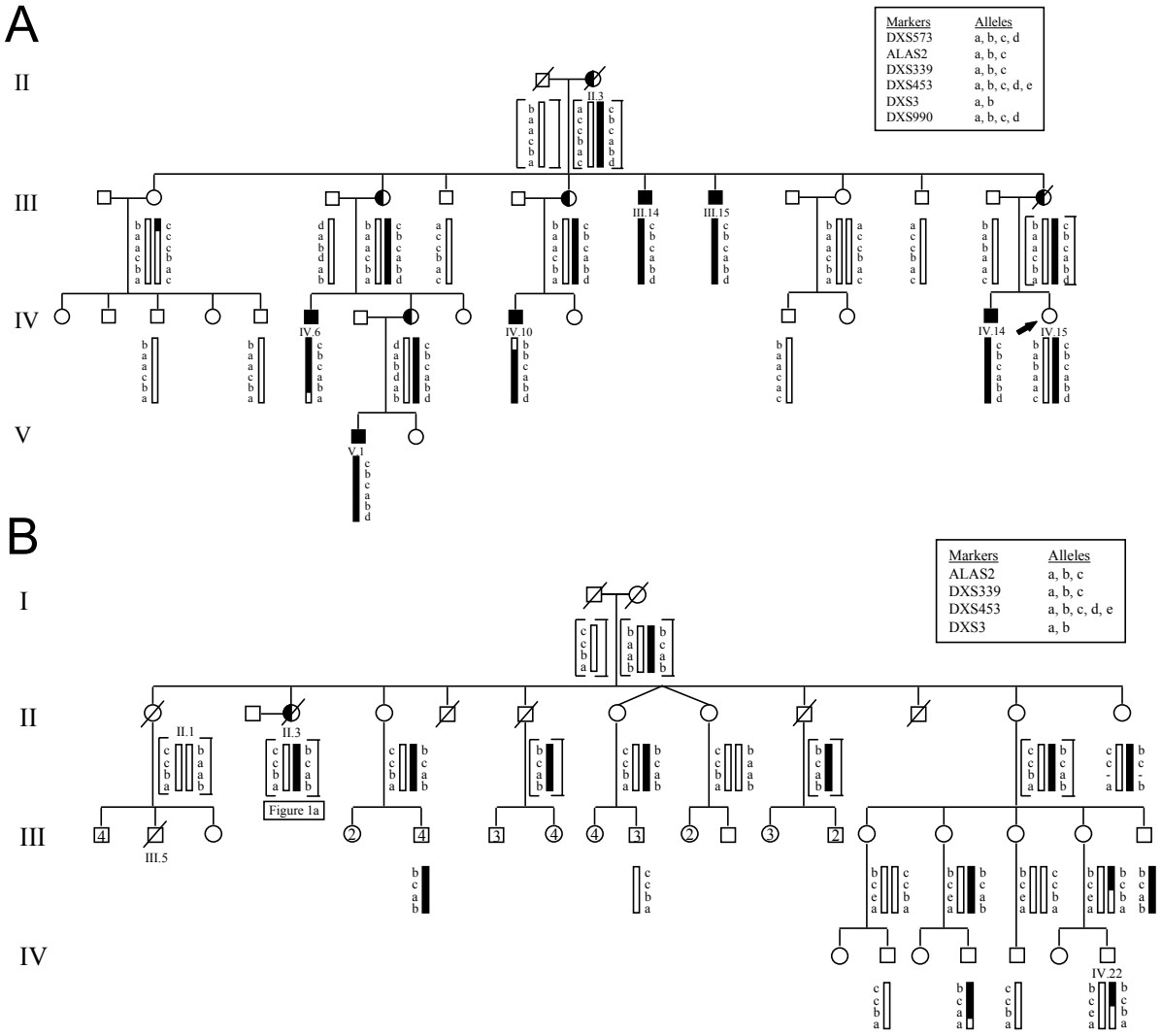

Figure 1. *Five-generation family with MRX. Fifty-two family members were available for linkage analysis.* ***A:*** *Original part of the family that is used for linkage analysis. Haplotypes of some of the linked markers in Xp11.3-q21.33 and the recombined markers delineating the probable gene location are shown. Deduced haplotypes from deceased individuals are represented between brackets. The disease chromosome is indicated by a black bar.* ***B:*** *Extended part of the family, showing that a recent mutation in the small branch has to be causative for the disease. Only the haplotypes for the linked markers from Fig. 1A are shown. The solid (III-5) and hatched (IV-22) symbols indicate that the mental retardation in these males is not due to mutation at the MRX65 locus. Obligate carrier females are indicated by half open circles.*

DNA analysis

DNA from 19 family members, isolated from peripheral blood lymphocytes according to the procedure of Miller *et al*.[12], was used for linkage analysis with 27 highly polymorphic markers distributed along the X-chromosome. Once linkage was established, 33 additional relatives were tested for the linked markers. Analysis of the markers involved amplification by polymerase chain reaction (PCR). Each reaction contained 100 ng genomic DNA and 30 ng of each primer, in 15 µl Supertaq buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 0.01% (w/v) gelatin) in the presence of 32^P-dCTP with 0.06 U Supertaq (HT Biotechnology LTD, Cambridge, England). Amplification was achieved by 35 cycles of 1 min 94°C, 2 min 55°C and 3 min 72°C with locus-specific primers registered in the Genome Database (http://gdbwww.gdb.org/). The radiolabeled PCR products were mixed with 15 µl sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue) heated to 95°C for 2 min and 4 µl of this mixture was separated on a 6.6% denaturing polyacrylamide gel. Subsequently, the gel was dried and exposed overnight to Kodak X-OMAT film to visualize the separated allelic bands.

Two-point linkage analyses of 27 polymorphic markers and the disease locus, tested in 19 family members, were performed with the MLINK option of the computer program LINKAGE (version 5.03)[13-15] on the basis of X-linked recessive inheritance with full penetrance. The relative order of marker loci was obtained from the Genome Database and the report of the Sixth International Workshop on X-Chromosome Mapping[16].

RESULTS

Results of clinical measurements and psychometric studies are summarized in Table 1. Pregnancy and delivery of patients IV-6, IV-10, IV-14, and V-1 were uneventful, whereas no information is available for patients III-14 and III-15. The mental impairment was noticed during early childhood and appeared nonprogressive. None of them had had convulsions and all had normal vision and hearing. All appeared to have relatively poor speech. Physically they were all healthy. Individual IV-10 was institutionalized and V-1 attended a special school for children with severe learning difficulties, while the others were living and working in a sheltered environment. Some showed chaotic behavior and aggressive outbursts. Only minor anomalies were noted in some of the affected males. Macrocephaly and obesity were observed in one and four patients, respectively. Small testes were observed in four of the patients (III-14, III-15, IV-6, and IV-10), but no exact measurements were available. The level of mental impairment varied from moderate to borderline. In all patients there was a tendency to score better in performance than in verbal intelligence tests. The male with borderline impairment (IV-14) had normal scores in performance tests, but showed mild mental retardation according to verbal tests. Since no consistent features other than mental retardation were seen, it was concluded that

pedigree structure and clinical data were fully compatible with a diagnosis of nonspecific X-linked mental retardation.

Table 1. *Summary of Clinical Measurements (centiles) and Psychometric studies*

Individual	Age (years)	Height (cm)	Weight (kg)	OFC[a] (cm)	Earlength (mm)	OCD[b] (cm)	ICD[b] (cm)	Mental Impairment[d]
III-14	56	173 (10)	100 (>97)	57 (50-90)	8 (>97)	8.5 (25-50)	2.5 (3)	mild
III-15	53	170 (3-10)	112 (>97)	58 (90)	8 (>97)	9 (50-75)	3 (50)	moderate
IV-6	52	177 (10-50)	90 (>97)	55.5 (50)	7 (75-97)	9.5 (75-97)	3 (50)	moderate
IV-10	24	162 (<3)	61 (10)	56.5 (50-90)	6.5 (75)	9 (50-75)	3 (50)	moderate
IV-14	28	181 (50)	96.5 (>97)	59 (>97)	7.5 (97)	9 (50-75)	2.6 (3-25)	borderline
V-1	6.5	106 (<3)	15 (<3)	47.5 (3)	4.5 (<3)	7.6 (25)	2.5 (3-25)	mild

[a]OFC, occipitofrontal circumference[17]
[b]OCD, outer canthal distance
[c]ICD, inner canthal distance
[d]Mental impairment: borderline, IQ 70-85; mild, IQ 55-69; moderate, IQ 40-54

For the initial analysis, markers chosen at regular distance on the whole X-chromosome were genotyped on 19 family members. Table 2 presents the results of the two-point linkage analysis in the small branch of the family (Figure 1A), between the MRX locus and each marker locus. Two regions with positive LOD scores were found. The relatively low LOD score in combination with haplotype analysis (data not shown) makes the localization of the genetic defect in the region between the MAO A and DXS6941 loci unlikely. Thus, the candidate region was defined by the proximal locus DXS573 at Xp11.3 and the more distal locus DXS990 at Xq21.33. A maximum LOD score of 3.64 was calculated for the markers ALAS2 and DXS453 at $\theta = 0$. A reconstruction of the haplotypes in the pericentromeric region is shown in Figure 1A.

In order to narrow down the linkage interval for MRX65, extensive genealogical studies were performed, which revealed an extended pedigree with two additional affected relatives (Figure 1B). Individual III-5 had already died. He was said to have attended special education school and to have suffered from perinatal asphyxia. Unfortunately no other data on this patient were available. Patient IV-22 was attending special education school because of learning disabilities.

Members from the extended part of the family were tested for the markers that showed linkage in the original branch. Haplotype analysis revealed seven unaffected males with the same haplotype in the linked region as the affected individuals in the original part of the family. This suggests that a new mutation occurred in II-3 and caused the disease in her six of here male descendants. Consequently, the reported mental impairment of III-5 and IV-22 has another etiology, in III-5 most likely the perinatal asphyxia. Interestingly, IV-22 DNA analysis clearly showed two allelic bands for most markers tested. Karyotype analysis in all 15 cells tested revealed that the patient has 48 chromosomes (48,XXXY). The extended part of the family was excluded from the linkage analysis. Haplotypes of the markers tested are shown in Figure 1B.

DISCUSSION

We have presented a new family (MRX65) with recessive nonspecific X-linked mental retardation and assigned the gene to a 45-cM interval between Xp11.3-q21.33, delimited by the markers DXS573 and DXS990.

Although some of the patients showed features that have also been reported in XLMR syndromes, these characteristics were found not to be consistent throughout the family. Macrocephaly, small testes, and obesity were reported in one, four, and four of the six affected males, respectively. Therefore, the disease segregating in this family is a nonspecific X-linked mental retardation condition, which makes it the twenty-sixth family linked to the pericentromeric region. In approximately half of the reported MRX families in which linkage studies are performed, the causative gene is located in this chromosomal segment. This suggests either a clustering of genes involved in X-linked mental retardation, or the existence of a few such genes that are frequently mutated. The results described in this paper provide evidence for a recent mutation in MRX65. An argument for a high mutation rate in a major MRX gene in this region is provided by the relatively small size of the linked families. Moreover, extensive genealogical studies that date back to the 18th century have failed so far to identify a common ancestor in the Nijmegen MRX families (APT Smits *et al.*, unpublished observations).

Table 2. *Lod Scores Between MRX65 and Markers Spread along the X-Chromosome (in order from Xpter to Xqter)*

Marker	0.000	0.050	2 0.100	0.200	0.300	0.400
DXS1060	- ∞	-0.22	0.42	0.76	0.67	0.38
DMD	- ∞	-2.78	-1.51	-0.49	-0.14	-0.08
DXS538	- ∞	-2.78	-1.51	-0.49	-0.14	-0.08
DXS7	-∞	-0.51	0.14	0.51	0.45	0.20
MAO A	-∞	1.05	1.35	1.32	0.96	0.45
DXS1003	1.74	1.62	1.48	1.16	0.77	0.32
DXS426	1.44	1.34	1.23	0.96	0.62	0.24
DXS6941	-∞	0.40	0.58	0.61	0.49	0.28
DXS573	-∞	2.05	2.05	1.71	1.18	0.54
ALAS2	3.64	3.37	3.08	2.43	1.67	0.79
DXS339	1.82	1.68	1.53	1.21	0.85	0.45
DXS453	3.64	3.37	3.08	2.43	1.67	0.79
DXS559	1.82	1.68	1.53	1.21	0.85	0.45
DXS986	2.04	1.88	1.71	1.32	0.87	0.36
DXS3	2.34	2.14	1.93	1.47	0.95	0.39
DXS990	-∞	1.23	1.32	1.15	0.80	0.34
DXS1231	-∞	2.07	2.08	1.76	1.23	0.57
DXS178	-∞	1.79	1.83	1.56	1.09	0.49
Col4A5	-∞	1.77	1.80	1.51	1.04	0.46
DXS424	-∞	0.78	0.90	0.80	0.53	0.19
DXS425	-∞	-2.42	-1.56	-0.76	-0.36	-0.12
HPRT	-∞	-5.84	-3.82	-1.93	-0.94	-0.35
DXS294	-∞	-3.69	-2.51	-1.36	-0.72	-0.30
DXS984	-∞	-2.20	-1.36	-0.60	-0.23	-0.06
FRAXAc2	-∞	0.12	0.32	0.40	0.34	0.20
DXS1113	-∞	-1.00	-0.70	-0.40	-0.22	-0.10
DXS1108	-∞	-1.78	-0.79	-0.02	0.23	0.22

The present study underlines the importance of the combination of clinical, cytogenetic, and molecular analysis. Due to the high prevalence of mental retardation in the general (male) population, extension of pedigrees might result in inclusion of false positive individuals (i.e., III-5 and IV-22) who were initially thought to be cases of nonspecific X-linked mental retardation. Only the combination of molecular and cytogenetic investigations revealed that their mental handicap was not due to a mutation at the MRX65 locus.

Note (added in proof): The family described here is the same as family F91-09 described in the article of Billuart *et al*. (1998). Although the *oligophrenin-1* gene is located within the linkage interval, no mutations were found in family MRX65.

REFERENCES

1. Lubs H, Chiurazzi P, Arena J, Schwartz C, Tranebjaerg L, and Neri G (1999) XLMR genes: update 1998. *Am J Med Genet* **83**: 237-247.
2. Gecz J, Gedeon AK, Sutherland GR, and Mulley JC (1996) Identification of the gene FMR2, associated with FRAXE mental retardation. *Nat Genet* **13**: 105-108.
3. D'Adamo P, Menegon A, Lo Nigro C, Grasso M, Gulisano M, Tamanini F, Bienvenu T, Gedeon AK, Oostra B, Wu S-K, Tandon A, Valtorta F, Balch WE, Chelly J, and Toniolo D (1998) Mutations in GDI1 are responsible for X-linked non-specific mental retardation. *Nat Genet* **19**: 134-139.
4. Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC, Zemni R, Roest Crollius H, Carrié A, Fauchereau F, Cherry M, Briault S, Hamel B, Fryns J-P, Beldjord C, Kahn A, Moraine C, and Chelly J (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**: 923-926.
5. American Psychiatric Association (1994) DSM-IV. 39-46. Washington DC: APA. p39-46.
6. Wechsler D (1974) Wechsler intelligence scale for children-revised. New York: Psychological Corporation.
7. van der Steene G, van Haasen PP, de Bruyn E.E.J., Coetsier P, Pijl YJ, Poortinga YH, Spelberg HC, Spoelders R, and Stinissen R (1986) Intelligence scale for children-revised. Lisse: Swets & Zeitlinger.
8. Bleichrodt N, Drenth PJD, Zaal J.M., and Resing WCM (1984) Revisie Amsterdamse kinder intellligentietest; instructie, normen, psychometrische gegevens. Lisse: Swets % Zeitlinger.
9. Kraijer DW and Kema GN (1994) Sociale redzaam-heidsschaal voor zwakzinnigen. Lisse: Swets & Zeitlinger.
10. Kraijer DW and Kema GN (1994) Sociale redzaam-heidsschaal voor zwakzinnigen van hoger nivo. Lisse: Swets & Zeitlinger.
11. Grossman HJ (1977) Manual on terminology and classification in mental retardation. Washington DC: AAMD.
12. Miller SA, Dykes DD, and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**: 1215-1215.
13. Lathrop GM and Lalouel JM (1984) Easy calculations of lod scores and genetic risks on small computers. *Am J Hum Genet* **36**: 460-465.
14. Lathrop GM, Lalouel JM, Julier C, and Ott J (1984) Strategies for multilocus linkage analysis in humans. *Proc Natl Acad Sci USA* **81**: 3443-3446.
15. Lathrop GM, Lalouel JM, and White RL (1986) Construction of human linkage maps: likelihood calculations for multilocus linkage analysis. *Genet Epidemiol* **3**: 39-52.
16. Nelson DL, Ballabio A, Cremers F, Monaco AP, and Schlessinger D (1995) Report of the sixth international workshop on X chromosome mapping 1995. *Cytogenet Cell Genet* **71**: 307-342.
17. Nellhaus G (1968) Head circumference from birth to eighteen years. Practical composite international and interracial graphs. *Pediatrics* **41**: 106-114.

Appendix 3

A novel ribosomal S6-kinase (*RSK4*; *RPS6KA6*) is commonly deleted in patients with complex X-linked mental retardation

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Published in: *Genomics* **62:** 332-343 (1999)

ABSTRACT

Large deletions in Xq21 often are associated with contiguous gene syndromes consisting of X-linked deafness type 3 (DFN3), mental retardation (MRX), and choroideremia (CHM). The identification of deletions associated with classic CHM or DFN3 facilitated the positional cloning of the underlying genes, *REP-1* and *POU3F4*, respectively, and enabled the positioning of the MRX gene in between these genes. Here, we report the cloning and characterization of a novel gene, ribosomal S6-kinase 4 (*RSK4*; HGMW-approved symbol *RPS6KA6*), which maps in the MRX critical region. *RSK4* is completely deleted in eight patients with the contiguous gene syndrome including MRX, partially deleted in a patient with DFN3 and present in patients with an Xq21 deletion and normal intellectual abilities. *RSK4* is most abundantly expressed in brain and kidney. The predicted protein of 746 amino acids shows a high level of homology to three previously isolated members of the human RSK family. *RSK2* is involved in Coffin-Lowry syndrome and nonspecific MRX. The localization of *RSK4* in the interval that is commonly deleted in mentally retarded males together with the high degree of amino acid identity with RSK2 suggests that RSK4 plays a role in normal neuronal development. Further mutation analyses in males with X-linked mental retardation must prove that *RSK4* is indeed a novel MRX gene.

INTRODUCTION

X-linked mental retardation (XLMR) is the most frequent genetic cause of mental handicap in males. A portion of all XLMR cases can be attributed to complex syndromes (MRXS), more than 100 of which have been described[1]. More often, the mental handicap is not associated with specific biochemical, neurological, or morphological abnormalities. This is referred to as nonspecific XLMR or MRX[2] and has an estimated incidence of 1 in 550 males[3]. At least 10 different genes are involved in the over 60 MRX families reported[1,4]. In only a minority of MRX cases, mutations are found in the recently identified four MRX genes: *FMR2*[5,6], *GDI1*[7,8], *OPHN1*[9], and *PAK3*[10]. The identification of other genes involved in MRX is hampered by the high genetic heterogeneity. The linkage intervals are generally too large to identify successfully the causative gene by positional cloning. New MRX genes can be identified either by candidate gene analysis, as has been achieved for *GDI1*[7,8] and *PAK3*[10], or by fine-mapping of the disease locus by using chromosomal abnormalities such as deletions or translocations. *FMR2* was identified because of its location next to a fragile site (FRAXE)[5,6], and a translocation breakpoint in Xq12 was used for the cloning of *OPHN1*[9]. Here, we describe the positional cloning of an MRX candidate gene in Xq21 by deletion mapping.

Deletions in Xq21 give rise to a complex phenotype including X-linked deafness type 3 (DFN3), MRX, and choroideremia (CHM)[11-13]. Sizable deletions have also been found in patients with nonsyndromic DFN3 or CHM[14-19] and in one patient with MRX and CHM[20].

Physical fine-mapping of the Xq21 deletions previously enabled us to clone the genes underlying CHM and DFN3, denoted *REP-1* and *POU3F4*, respectively[21-23]. The gene underlying MRX in patients with this contiguous gene syndrome has been mapped to a region between *POU3F4* and *REP-1*[13,20]. We have narrowed down the MRX critical region in Xq21 by fine-mapping the deletion in patients with DFN3. Cosmids derived from a yeast artificial chromosome (YAC) spanning the critical interval were used to isolate a novel gene, named *RSK4**. To demonstrate the involvement of this gene in MRX, we searched for mutations in 200 families with multiplex cases of mental retardation compatible with X-linked inheritance, employing single-stranded conformation polymorphism (SSCP) analysis and direct sequencing.

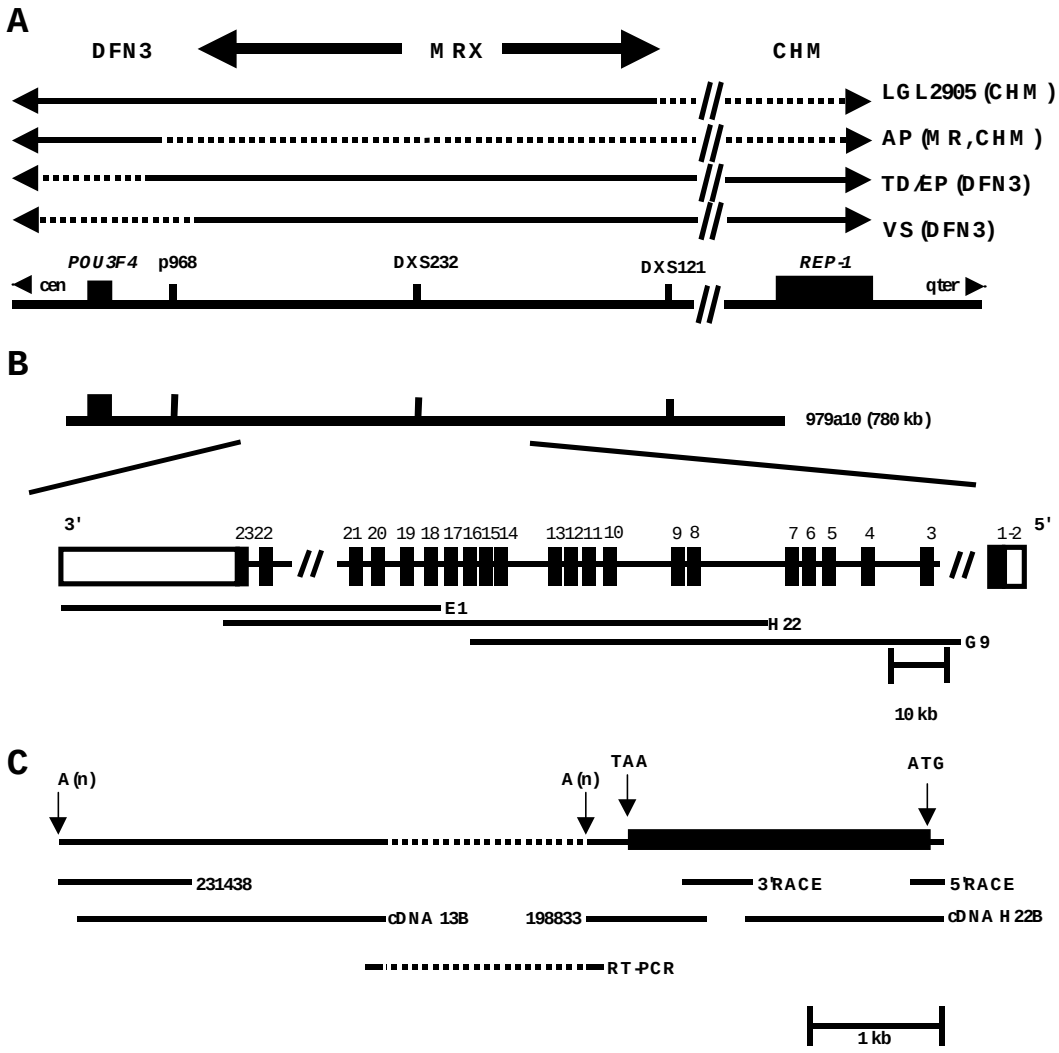

Figure 1. *Physical map of the Xq21 region containing the MRX locus and the RSK4 gene.* ***A:*** *Patients with deletions (represented by dotted lines) that have been critical for narrowing the MRX critical interval are represented. LGL2905[18] suffers from CHM; AP[20] suffers from CHM and MR; and patients TD[14], EP, and VS[16] suffer from DFN3.* ***B:*** *The RSK4 gene spans approximately 75 kb. Marker p968 is present on cosmid E1; marker DXS232 is mapped on cosmid G9. The intron sizes are drawn to scale.* ***C:*** *The total RSK4 cDNA is represented by two RACE clones, two clones from a retina cDNA library (13B and H22), an RT-PCR product, and two clones from the GenBank database (Accession Nos. 231438 (pineal gland) and 198833 (fetal liver/spleen).*

* The HGMW-approved symbol for the gene described in this paper is *RPS6KA6*.

MATERIALS AND METHODS

Patient material

Patients with a deletion in Xq21 have been described previously (reviewed in Philippe *et al*.[24]). DNA from CHM patient LGL2905[18], patient AP[20] who suffers from CHM and MR, and DFN3 patients TD[14], EP, and VS[16] has been examined for narrowing down the MRX critical interval. Clinical data and blood samples of MRX(S) families tested in SSCP analysis were obtained after informed consent. DNA from peripheral blood lymphocytes was isolated according to the procedure of Miller *et al*.[25]. Affected males of 200 families were tested, 16 of which had a genetic defect linked to Xq21. Some of these families have been described: MRX4[26], MRX13[27], MRX20[28], MRX52[29], MRX65[30], Wilson-Turner syndrome[31], MR with ataxia and deafness[32], and MR with cleft palate and short stature[33].

Physical map of the MRX locus

Fine-mapping of the region surrounding the MRX locus was performed by analyzing breakpoints of deletions in the Xq21 region. DNA from patients LGL2905, AP, TD, EP and VS was analyzed by PCR analysis (DXS995, DXS232, and DXS121) and Southern blotting (p968). p968 was generated as a 2.3-kb inter-Alu PCR product with the CotterIV primer[34]. A partial cosmid contig from the region was obtained by subcloning YAC 970a10 from the CEPH library using the sCOGH2 vector[35].

Isolation of RSK4 cDNA clones

Human cDNA clone 231438 (GenBank Accession No. H92571 (3′) and H92239 (5′)) was mapped within the MRX critical interval by PCR analysis on a panel of patients with a deletion in Xq21[24]. This cDNA was radioactively labeled by random primer extension[36] and used to screen 1 x 106 plaques of a human embryonic craniofacial cDNA library in Lambda ZAP II (obtained from Dr. J. Murray) at 100,000 plaques/150-mm dish by standard protocols using Hybond-N+ membranes. Hybridizations were performed at 65°C overnight in 0.125 M PO_4, 0.25 M NaCl, 1 mM EDTA, 7% (w/v) SDS, 10% (w/v) PEG 6000. Positive clones were purified by secondary and tertiary screening using nitrocellulose filters and NC hybridization buffer (6x SSC, 5x Denhardt's, 0.2% (w/v) SDS, 10% (w/v) dextrane sulfate). Positive phages were converted to plasmid by in vivo excision according to the manufacturer's protocol (Stratagene). Single colonies were grown overnight at 37°C, and DNA isolation was performed using a Qiaprep 8 purification kit (Qiagen). Sequence PCRs were performed using the Taq Dye Terminator Cycle Sequencing Ready reaction kit (Pharmacia). Sequences were analyzed on an ABI automated DNA sequencer.

One of the isolated cDNA clones as well as cosmid DNA was used to screen 1x106 plaques of a retina cDNA library (Clontech, HL 1132a) in λgt10 at 80,000 plaques/150-mm dish, following the same hybridization protocols. Phage DNA of positive plaques was

isolated using the Lambda miniprep kit (Qiagen), and inserts were purified and sequenced. Additional sequence was also obtained from human fetal brain Marathon-Ready cDNA (Clontech) by 3′ rapid amplification of cDNA ends (RACE) and 5′ RACE, following the enclosed protocol.

To prove that the isolated cDNAs belong to the same gene, (RT-) PCR analysis was performed on DNA and RNA isolated from a lymphoblastoid cell line of a healthy individual. For amplification, a forward primer in the RSK4 cDNA (5′-ggggaagtgagatttaaaaaacc-3′) and a reverse primer in the cDNA corresponding to the 3′ noncoding region (5′-accaaagttacgagtttcctag-3′) were used.

Expression of RSK4 cDNA

Northern blots containing poly(A)+ RNA from different fetal and adult tissues (Clontech MTN, 7760-1) and a Northern dot-blot (human RNA master blot, Clontech, 7770-1) were hybridized with radioactively labeled probes made by random primer extension of cDNA clones 231438 and H22B, following the Clontech protocol.

Identification of the genomic structure of RSK4

Intronic sequences of RSK4 were determined by long-range amplification using ExTaq (TaKaRa Biomedicals) with exon-specific primers and sequencing of the subsequent PCR products. Introns that appeared too large for long-range PCR were directly sequenced on DNA from cosmid E1 or H22 using exon-specific primers. All primer sequences are available upon request.

Mutation detection

PCR-SSCP analysis was performed according to the method of Orita *et al*.[37], with slight modifications. Oligonucleotides used for genomic PCR amplification of 21 RSK4 exons are listed in Table 2. In all PCR experiments γ-32^{P}-end-labeled primers were used as described by Van den Hurk *et al*.[38]. All PCRs were performed in a PTC-100 Programmable Thermal Controler (MJ Research, Inc.), with cycling conditions of 95°C for 1 min, 55 or 58°C (listed in Table 2) for 2 min, and 72°C for 1 min, for 30 cycles. Samples of the reaction were denatured and electrophoresed on a 5% nondenaturing polyacrylamide gel[38]. PCR fragments with an altered electrophoretic mobility were sequenced.

RESULTS

Fine-Mapping of the MRX Critical Interval

The physical map of the Xq21 region by Colleaux *et al*.[39] shows that YAC clone 979a10 of the CEPH Mega-YAC library contained the markers DXS995, DXS232, and DXS121. Although of our YAC 979a10 isolate is smaller than the original clone (780 instead

of 1600 kb), the same markers could be PCR-amplified (data not shown). This indicates that YAC 979a10 spans the MRX critical interval including the gap in the Xq21 YAC contig constructed by Van der Maarel *et al*.[20].

To narrow down the MRX critical interval, the extent of the deletion in patients LGL2905, AP, TD, EP, and VS was investigated by Southern blotting and PCR amplification of several markers in Xq21. Hybridization of a Southern blot, containing *Eco*RI-digested genomic DNA, with a probe derived from a 2.3-kb inter-Alu PCR product of YAC 979a10 (p968) showed that this marker was not present in DFN3 patient VS (data not shown). The marker therefore defines the proximal boundary of the MRX critical interval. PCR amplification of marker DXS121 revealed that it was absent in CHM patient LGL2905 (data not shown), indicating that the MRX gene is located proximal of this marker. The MRX critical interval could therefore be assigned between markers p968 and DXS121 (Figure 1).

Cloning and Sequencing of a Candidate Gene for MRX

A panel of males with deletions in Xq21[24] was used for the fine-mapping of ESTs that had been roughly assigned to this chromosomal region. For this purpose, DNA of the patients was PCR-amplified with EST-specific primers. One of the clones (human cDNA clone 231438) appeared to map exactly within the MRX critical region (Figure 1) (data not shown). This cDNA clone was used to screen a human embryonic craniofacial cDNA library (obtained from Dr. J. Murray) and a retina cDNA library. Eleven partially overlapping clones were obtained, together spanning 2540 bp. The sequence did not contain an open reading frame (ORF). These cDNA clones were mapped to cosmid E1, which was derived by subcloning YAC 979a10 into cosmid vector sCOGH2. As these cDNAs did not hybridize to additional cDNA clones, we decided to use an adjacent cosmid, H22, to screen the retina cDNA library. Three partially overlapping cDNA clones were obtained, which together with a 3′ RACE experiment and cDNA clone 198833, allowed the construction of a 2640-bp sequence, containing a 2238-bp ORF of a novel gene. No overlap was observed between this cDNA sequence and the 2540-bp 3′ noncoding cDNA sequence. To investigate whether the cDNAs belong to the same gene, PCR analysis was performed using primers in both cDNAs. PCR amplification of DNA and RNA (by RT-PCR) extracted from a lymphoblastoid cell line of a control individual revealed fragments of the same size (approximately 1.5 kb), indicating that the two cDNAs are indeed part of the same gene.

The putative start codon was found in only one cDNA clone, which contains an unusual T-stretch in the 5′ untranslated region. To confirm this sequence, we performed a 5′ RACE experiment on cDNA derived from human fetal brain. Six different clones were sequenced and found to match the sequence of the cDNA clone from the retina cDNA library. The size of the T-stretch varied between 7 and 50 nucleotides in the various clones. The complete RSK4 cDNA and deduced amino acid sequence are shown in Figure 2.

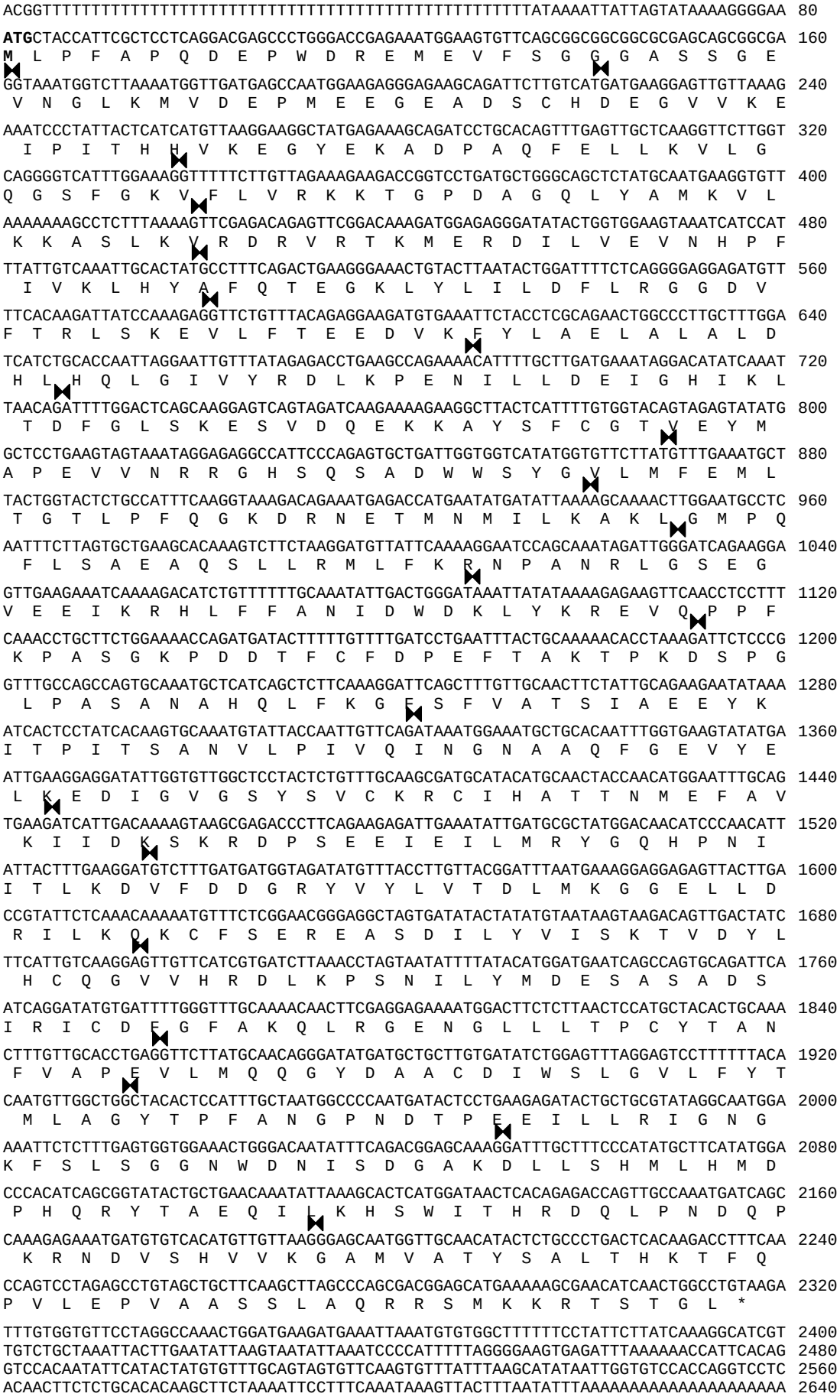

***Figure 2**. Nucleotide sequence of the RSK4 cDNA with deduced amino acid sequence below. Arrowheads show exon-exon junctions. An asterisk indicates a stopcodon at position 2316-2318. The cDNA and deduced protein sequence are in GenBank (Accession No. AF184065).*

```
RSK1   --MPLAQLKEPW------PLMELVPLDPENGQTSG-----------EEAGLQPSKDEGVLKEISITHHVKA
RSK2   --MPLAQLADPW----QKMAVESPSDSAENGQQIMDEPMG-------EEEINPQTEEVSIKEIAITHHVKE
RSK3   --MDLSMKKFAVR-----R-FFSVYLRRKSRSKSS-------------SLSRLEEEGVVKEIDISHHVKE
RSK4   -MLPFAPQDEPWDREMEVFSGGGASSGEVNGLKMVDEPME-------EGEADSCHDEGVVKEIPITHHVKE
         .  .                                                .*  . *** *.****

RSK1   GSEKADPSHFELLKVLGQGSFGKVFLVRKVTRPDSGHLYAMKVLKKATLKVRDRVRTKMERDILADVNHPF
RSK2   GHEKADPSQFELLKVLGQGSFGKVFLVKKISGSDARQLYAMKVLKKATLKVRDRVRTKMERDILVEVNHPF
RSK3   GFEKADPSQFELLKVLGQGSYGKVFLVRKVKGSDAGQLYAMKVLKKATLKVRDRVRSKMERDILAEVNHPF
RSK4   GYEKADPAQFELLKVLGQGSFGKVFLVRKKTGPDAGQLYAMKVLKKASLKVRDRVRTKMERDILVEVNHPF
       * *****..**********.******.*    *. .**********.********.******* .*****

RSK1   VVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALGLDHLHSLGIIYRDLKPENIL
RSK2   IVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALALDHLHSLGIIYRDLKPENIL
RSK3   IVKLHYAFQTEGKLYLILDFLRGGDLFTRLSKEVMFTEEDVKFYLAELALALDHLHSLGIIYRDLKPENIL
RSK4   IVKLHYAFQTEGKLYLILDFLRGGDVFTRLSKEVLFTEEDVKFYLAELALALDHLHQLGIVYRDLKPENIL
       .************************.********.*************** ***** ***.**********

RSK1   LDEEGHIKLTDFGLSKEAIDHEKKAYSFCGTVEYM**APE**VVNRQGHSHSADWWSYGVLMFEMLTGSLPFQGK
RSK2   LDEEGHIKLTDFGLSKESIDHEKKAYSFCGTVEYM**APE**VVNRRGHTQSADWWSFGVLMFEMLTGTLPFQGK
RSK3   LDEEGHIKITDFGLSKEAIDHDKRAYSFCGTIEYM**APE**VVNRRGHTQSADWWSFGVLMFEMLTGSLPFQGK
RSK4   LDEIGHIKLTDFGLSKESVDQEKKAYSFCGTVEYM**APE**VVNRRGHSQSADWWSYGVLMFEMLTGTLPFQGK
       *** ****.********..*..*.*******.**********.**..******.**********.******

RSK1   DRKETMTLILKAKLGMPQFLSTEAQSLLRALFKRNPANRLGSGPDGAEEIKRHVFYSTIDWNKLYRREITP
RSK2   DRKETMTMILKAKLGMPQFLSPEAQSLLRMLFKRNPANRLGAGPDGVEEIKRHSFFSTIDWNKLYRREIHP
RSK3   DRKETMALILKAKLGMPQFLSGEAQSLLRALFKRNPCNRLGAGIDGVEEIKRHPFFVTIDWNTLYRKEIKP
RSK4   DRNETMNMILKAKLGMPQFLSAEAQSLLRMLFKRNPANRLGS--EGVEEIKRHLFFANIDWDKLYKREVQP
       ** *** .************* ******* ******.****.  .* ****** *. .***  **..*. *

RSK1   PFKPAVAQPDDTFYFDTEFTSRTPKDSPGIPPSAGAHQLFRGFSFVATGLMEDDGKPRAPQAPLHSVVQQL
RSK2   PFKPATGRPEDTFYFDPEFTAKTPKDSPGIPPSANAHQLFRGFSFVAITSDDES--QAMQTVGVHSIVQQL
RSK3   PFKPALGRPEDTFHFDPEFTARTPTDSPGVPPSANAHHLFRGFSFVASSLIQEPSQQDLHKVPVHPIVQQL
RSK4   PFKPASGKPDDTFCFDPEFTAKTPKDSPGLPASANAHQLFKGFSFVATSIAEEYKITPITSANVLPIV-QI
       *****  .*.*** ** ***..** ****.* **.**.**.******     .         .  .* *.

RSK1   HGKNLVFSDGYVVKETIGVGSYSECKRCVHKATNMEYAVKVIDKSKRDPSEEIEILLRYGQHPNIITLKDV
RSK2   HRNSIQFTDGYEVKEDIGVGSYSVCKRCIHKATNMEFAVKIIDKSKRDPTEEIEILLRYGQHPNIITLKDV
RSK3   HGNNIHFTDGYEIKEDIGVGSYSVCKRCVHKATDTEYAVKIIDKSKRDPSEEIEILLRYGQHPNIITLKDV
RSK4   NGNAAQFGEVYELKEDIGVGSYSVCKRCIHATTNMEFAVKIIDKSKRDPSEEIEILMRYGQHPNIITLKDV
       .     *  .  *  .** ******* ****.*  .*  *.***.********.******.**************

RSK1   YDDGKHVYLVTELMRGGELLDKILRQKFFSEREASFVLHTIGKTVEYLHSQGVVHRDLKPSNILYVDESGN
RSK2   YDDGKYVYVVTELMKGGELLDKILRQKFFSEREASAVLFTITKTVEYLHAQGVVHRDLKPSNILYVDESGN
RSK3   YDDGKFVYLVMELMRGGELLDRILRQRYFSEREASDVLCTITKTMDYLHSQGVVHRDLKPSNILYRDESGS
RSK4   FDDGRYVYLVTDLMKGGELLDRILKQKCFSEREASDILYVISKTVDYLHCQGVVHRDLKPSNILYMDESAS
.***. **.* .**.******.**.*. *************  *  * **..***.*************** ***

RSK1   PECLRICDFGFAKQLRAENGLLMTPCYTANFV**APE**VLKRQGYDEGCDIWSLGILLYTMLAGYTPFANGPSD
RSK2   PESIRICDFGFAKQLRAENGLLMTPCYTANFV**APE**VLKRQGYDAACDIWSLGVLLYTMLTGYTPFANGPDD
RSK3   PESIRVCDFGFAKQLRAGNGLLMTPCYTANFV**APE**VLKRQGYDAACDIWSLGILLYTMLAGFTPFANGPDD
RSK4   ADSIRICDFGFAKQLRGENGLLLTPCYTANFV**APE**VLMQQGYDAACDIWSLGVLFYTMLAGYTPFANGPND
        ...*.**********  ****.************** .****  *******.* ****.*.******* *

RSK1   TPEEILTRIGSGKFTLSGGNWNTVSETAKDLVSKMLHVDPHQRLTAKQVLQHPWVTQKDKLPQSQLSHQDL
RSK2   TPEEILARIGSGKFSLSGGYWNSVSDTAKDLVSKMLHVDPHQRLTAALVLRHPWIVHWDQLPQYQLNRQDA
RSK3   TPEEILARIGSGKYALSGGNWDSISDAAKDVVSKMLHVDPHQRLTAMQVLKHPWVVNREYLSPNQLSRQDV
RSK4   TPEEILLRIGNGKFSLSGGNWDNISDGAKDLLSHMLHMDPHQRYTAEQILKHSWITHRDQLPNDQPKRNDV
       ****** *** **..**** *  .*. ***..*.***.***** **  .*.* *.   . *   *  ..*

RSK1   -QLVKGAMAATYSALNSSKPTPQLKPIESSILAQRR-VRKLPSTTL
RSK2   PHLVKGAMAATYSALNRN-QSPVLEPVGRSTLAQRRGIKKITSTAL
RSK3   -HLVKGAMAATYFALNRTPQAPRLEPVLSSNLAQRRGMKRLTSTRL
RSK4   SHVVKGAMVATYSALTHKTFQPVLEPVAASSLAQRRSMKKRTSTGL
        ..***** *** **.     * * *.  * ***** ...  ** *
```

Figure 3. *Alignment of human RSK proteins. The conserved kinase domains ($GQGSFGX_{19}K$ and $GVGSYSX_{16}K$) are shown in boxes, and the APE motifs are highlighted in boldface type. Asterisk; amino acid identity; dot; amino acid similarity. Alignment was carried out using the Clustal W program (version 1.5). Amino acid sequences are translations from the deposited cDNA sequences in GenBank (human RSK1, Accession No. L07597; human RSK2, Accession No. L07598; human RSK3, Accession No. L07599; and human RSK4, Accession No. AF184965).*

Homology of the Novel Gene to Human RSKs

A FASTA search[40] revealed a strong homology between the novel gene and genes of the ribosomal S6 kinase (RSK) family of different species. Comparison of the coding nucleotides and the deduced amino acid sequence of the novel gene with the three known human *RSK* genes clearly showed that we have identified a novel member of this family, which we have designated *RSK4* (Figure 3).

Expression of the *RSK4* Gene

Northern blots containing poly(A)+ RNA from a number of fetal and adult tissues were hybridized with the insert of a retina cDNA clone containing the first 17 exons of RSK4 (H22B, Figure 1). Three transcripts of approximately 5, 6.5, and 9 kb were detected, with the 6.5-kb band being the most prominent. The expression is most abundant in fetal (data not shown) and adult kidney and brain and lower in all other tissues tested (Figure 4A). To prove that these signals were not due to cross-hybridization with the other RSK genes, the Northern blots were also hybridized with cDNA clone 231438 from the 3′ noncoding region. The same three transcripts were found, which added further evidence to the earlier finding that the RSK4 cDNAs and the 3′ NCR are derived from the same gene. The total cDNA clone has a length of approximately 6.5 kb, which corresponds to the most prominent band on the Northern blot. The other (minor) transcripts are probably caused by alternative splicing or alternative polyadenylation. Hybridization of a multiple tissue Northern dot-blot showed highest expression in fetal kidney, pituitary, and thyroid gland (Figure 4B).

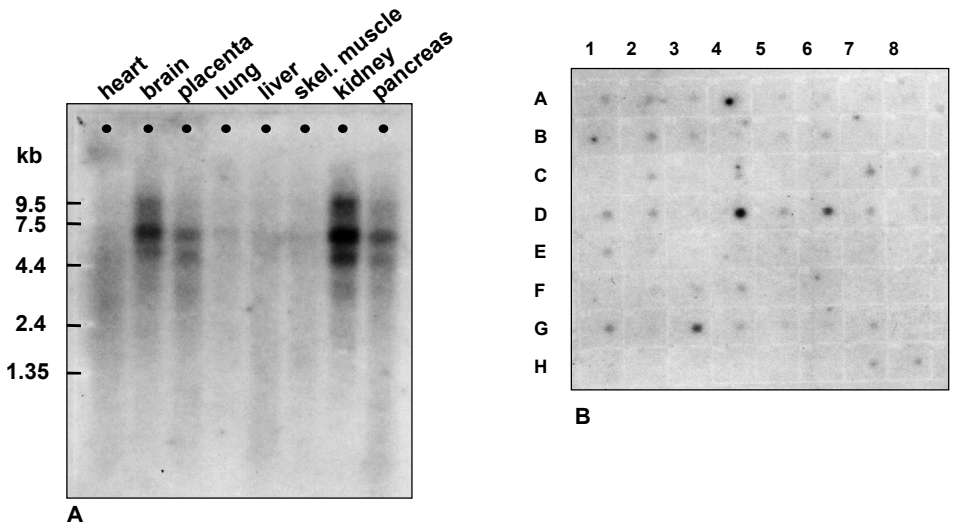

Figure 4. *Northern blot analysis of RSK4 expression. cDNA clone H22B (Fig 1.) was used as a probe.* ***A:*** *Adult multiple tissue Northern blot containing poly(A)$^+$ RNA. Transcripts of approximately 5, 6.5, and 9 kb were detected with highest expression in kidney and brain tissue.* ***B:*** *Multiple tissue Northern dot-blot showing highest expression in pituitary gland (D4). RSK4 is also expressed in thyroid gland (D6) and fetal kidney (G3). The dot in section A4 is a hybridization artifact just beside the spotted RNA, which was not seen in duplicate hybridizations.*

Table 1. *Splice sites of the RSK4 gene*

Exon	Splice acceptor site	Con-sensus	Splice donor site	Con-sensus	Position	Exon size (bp)	Intron size (kb)
1	-	-	-			-	-
2	-	-	-		162	-	-
3	acaatgtttttcagGTAAATGGTC	85%	TTCTTGTCATgtaagt	87%	221	59	±8
4	atttctttttacagGATGAAGGAG	93%	ATTTGGAAAGgtaatg	81%	338	117	±7
5	caattttctattagGTTTTTCTTG	90%	TCTTTAAAAGgtagaa	69%	420	82	±0.8
6	tatgtaatttttagTTCGAGACAG	73%	TTGCACTATGgtatgt	81%	501	81	±1
7	taatttttaattagCCTTTCAGAC	72%	ATCCAAAGAGgtaagt	10%	581	80	±15
8	ttattttgttctagGTTCTGTTTA	90%	AGCCAGAAAAgtaaag	69%	688	107	0.238
9	attttgatttacagCATTTTGCTT	89%	AAATTAACAGgtatgc	83%	726	38	±10
10	tactgttattatagATTTTGGACT	79%	TGTTCTTATGgtaagt	92%	869	143	±2
11	tttgtttgttttagTTTGAAATGC	84%	TGATATTAAAgtaagt	88%	940	71	0.235
12	atcctctgtttcagAGCAAAACTT	89%	AATAGATTGGgtattg	62%	1029	89	±1.5
13	tctttttttttatagGATCAGAAGG	91%	TGACTGGGATgtaagt	87%	1088	59	±7
14	gttgtcccttctagAAATTATATA	85%	ACACCTAAAGgtaatg	81%	1191	103	±1
15	tatttggacattagATTCTCCCGG	70%	AATTGTTCAGgtaaat	86%	1322	131	0.423
16	ttaaaaataaacagATAAATGGAA	65%	TGCAGTGAAGgtattg	70%	1445	123	±1
17	ttcgtatttcttagATCATTGACA	83%	TTTGAAGGATgtaggt	77%	1535	90	±1.5
18	gcgttctttttgtagGTCTTTGATG	87%	TTGTCAAGGAgtaagt	80%	1694	159	±2
19	cctttatattcaagGTTGTTCATC	84%	TGCACCTGAGgtatcc	69%	1856	162	±3
20	tatttctttcttagGTTCTTATGC	90%	TGTTGGCTGGgtaaga	87%	1933	77	±1.8
21	tattttattaatagCTACACTCCA	80%	CGGAGCAAAGgtataa	69%	2051	118	-
22	ttttttttaatttagGATTTGCTTT	91%	TGTTGTTAAGgtaaaa	80%	2192	141	±0.400
23	tctcattactttagGGAGCAATGG	82%	-	-	-	-	-

Note. Lowercase letters indicate intron sequences; uppercase letters indicate exon sequences. The percentage similarity to splice consensus sequences was calculated according to Shapiro and Senapathy[41]. The position of the splice sites on *RSK4* cDNA is as reflected in Figure 2.

Genomic Structure of RSK4

Cosmid sequencing and long-range PCR amplification of genomic DNA allowed us to identify 21 introns interrupting the *RSK4* coding region. Despite repeated trials with different primers in the 5' part of the gene, no specific PCR product could be generated on genomic DNA, indicating that probably one or more large introns are present. Hence, the gene consists of at least 22 exons that span approximately 75 kb of genomic DNA and are located on cosmids E1, H22, and G9 in a telomeric to centromeric orientation (Figure 1). All splice donor and splice acceptor sites fit the consensus as reported by Shapiro and Senapathy[41]. The sequences of the intron-exon boundaries, as well as exon and intron sizes, are given in Table 1. PCR analysis of different exons revealed that patient AP, who suffers from CHM and MRX, had a deletion of the entire *RSK4* gene. Patients TD and EP (patients with DFN3) and LGL2905 (patient with CHM) contained the entire *RSK4* gene. Patient VS, however, appeared to have a deletion of the last 14 exons, although he is diagnosed as a nonsyndromic DFN3 patient (Figure 5). Recently performed tests revealed that this patient is of normal intelligence (Dr. H. Heilbronner and Dr. H.-J. Pander, pers. comm.). These results indicate that 9 of the 23 *RSK4* exons are located within the MRX critical interval (Figure 1).

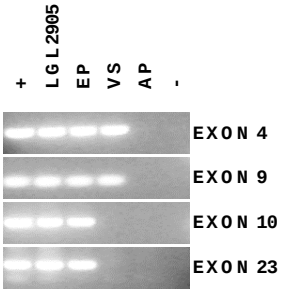

Figure 5. *PCR amplification of RSK4 exons 4,9, 10, and 23 with primer sets described in Table 2. +; DNA from a healthy individual (positive control), -; no DNA template (negative control).*

Table 2. *Oligonucleotides used for PCR amplification of RSK4 exons*

Exon	Oligonucleotide (5' → 3')	Orientation	Code	Size (bp)	$MgCl_2$ concentration(mM)	T_a(°C)
1	-	-		-		-
2	-	-		-		-
3	CACTCTTGTTTTAATACTTTTGAAC	Sense	2365	189	3	58
	GGAGCTTTTCTCCTTTCCCTG	Antisense	2353			
4	CAGAAGATGTTGAAAATGGATTC	Sense	2354	245	3	58
	CAGATTTCTACGTATGTGTTCAC	Antisense	2355			
5	GAGCAAGAATGAGTAGAAATGTG	Sense	2327	235	3	58
	GCTACATCTATTCACCTTATGC	Antisense	2129			
6	GGCATTTAGGAAGTTACGTTTTG	Sense	2285	281	1.5	55
	GTGTGAAAGGAAATCATAATTCTC	Antisense	2130			
7	GTTATCCATTAATGAACCATGTG	Sense	2131	281	3	55
	GTAATTAGCAGAAGAACAAAAGTG	Antisense	2132			
8	GTCTGTCTACTCATTTTGAAAG	Sense	2133	255	4.5	58
	GTTTGACAAAAAGCAGTTCCAG	Antisense	2386			
9	GTTTTATGTAAAATATTTTATTCCATC	Sense	2409	128	4.5	55
	CAAGTGGCTAAGGGTATAATAAG	Antisense	2449			
10	CGTTTTCTATTTTCTGAAGTTTTTC	Sense	2398	249	3	55
	GTAATATCAATCACAGGTTGGC	Antisense	2390			
11	CTATCATGTAGATCTTACAATTTG	Sense	2385	297	4.5	55
	CAGTGAGAAAAAGGTACTCTTG	Antisense	2134			
12	GAGATTTTTCAAATCAAAAATGTGG	Sense	2135	194	3	55
	CATGCAGTTACTTCTATAAAAGC	Antisense	2328			
13	GGAATGTATGAAATATGTGTATTC	Sense	2646	262	3	55
	CCACTGATTTGTTATTTAGATTAAAAG	Antisense	2647			
14	CTTAATACGTTTTGTGTTATCTGG	Sense	2371	187	4.5	55
	CTATCTACTAAATAAAATCTCTG	Antisense	2287			
15	CCTTTATAGTTAAAGAAATTCTGAC	Sense	2286	272	3	55
	GCAATAACAGAGAAATAATTATCC	Antisense	2300			
16	GGCTTAAGATGAATGTATATTTAG	Sense	2136	289	3	58
	GAAATGGTTGTCAGTTGACTC	Antisense	2368			
17	GACAAAATAATTTGTTTGCCTC	Sense	2391	212	4.5	55
	CAATAAGAAAAATAAAGTTCCAATTC	Antisense	2394			
18	GTAGAAAGGTAAATATAATTAAAATC	Sense	2372	297	4.5	55
	GAAGATATAGGTCCCAGAATAG	Antisense	2303			
19	GTAATTTGGAAAGTTTCTTTTTCTC	Sense	2289	343	3	55
	GGCATCATATAGATTCAACTAAAG	Antisense	2373			
20	GGAGAAAATTGAGTTTATATTGAG	Sense	2392	204	4.5	55
	CTCTTTCTAGATGAAGATATACAG	Antisense	2399			
21	GTTTGGGTATGTTACCCTCTAG	Sense	2393	276	4.5	55
	CACTTTACATGACACCATTAG	Antisense	2450			
22	GCTGTCACATTTTCAGCAAATG	Sense	2412	274	3	55
	GGGTTTATTAGGTATCTGATATC	Antisense	2410			
23	CTCCAGAATTTGTACTTAGTTTG	Sense	2401	242	3	55
	GATAAGAATAGGAAAAAAGCCAC	Antisense	2414			

Note. The fragment size of the PCR products, as well as the $MgCl_2$ concentration and the annealing temperature (T_a) by which the primer pairs are used in PCR-SSCP analysis is indicated.

Mutation Analysis of RSK4 as a Candidate Gene for MRX

We analyzed DNA from 200 unrelated male MRX patients for mutations in RSK4. Primer sets in the intronic sequences were used in PCR-SSCP analysis (listed in Table 2). In this analysis, DNA of 13 MRX and 3 MRXS families in which the molecular defect was genetically linked to the Xq21 region was included. PCR-amplified fragments with altered electrophoretic mobility were further analyzed by direct DNA sequencing. We identified a G-to-A transition at position 2154 in 8 of the 200 patients, changing an aspartic acid (GAT) to an asparagine (AAT) (D692N). Hybridization with an allele-specific oligonucleotide revealed this amino acid substitution in 7 of 88 healthy individuals, indicating that it is a polymorphism rather than a mutation. We also identified three different intronic alterations. Although none of these sequence changes has an effect on the calculated splice efficiency as shown in Table 1, a panel of control persons has been tested. Since all intronic changes have been found in at least one control individual, these variations can be considered as polymorphisms. All sequence changes found are listed in Table 3.

Table 3. *Polymorphisms in the RSK4 Gene*

Sequence change	Incidence in MRX males	Incidence in controls
IVS6-28C>T	1/200	1/157
IVS13+15A>C	2/200	3/80
IVS17-63-59del5	1/200	2/145
2154G>A	8/200	7/88

Note. The nomenclature is according to Antonarakis *et al.*[42]. The IVS17-63-59del5 change was also found in two unaffected males in the same family (apart from the two control persons mentioned in this table). The position of the 2154G>A polymorphism (D692N) is as reflected in Figure 2.

DISCUSSION

Under the assumption that contiguous gene syndromes associated with Xq21 deletions consist of three separate disease entities, i.e., DFN3, MRX, and CHM, we have employed a positional cloning approach to identify the elusive Xq21 MRX gene. We identified a novel gene, RSK4, which is deleted in patients with DFN3, MRX, and CHM as well as in one patient with MRX and CHM. With one exception, all other deletions investigated do not involve the RSK4 gene, and, accordingly, these males are not mentally retarded. Only DFN3 patient VS (Figure 1) lacks exons 10-23 of the RSK4 gene, but is not mentally retarded.

RSK4 is a novel member of a family of growth-factor-related serine-threonine kinases, known as p90rsk or RSK (for ribosomal S6 kinase)[43]. In humans, three closely related members of the RSK family have previously been isolated: *RSK1*, *RSK2* and *RSK3*[44,45]. A highly conserved feature of the RSK protein family is the presence of two nonidentical, kinase catalytic domains that are each shared by other distinct protein kinases[46]. The p90rsk protein kinases are activated by multisite serine-threonine phosphorylation in response to a variety of growth factors and hormones[47-52]. Several lines of evidence suggest that mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinases (ERK-1 and -2) and c-Jun amino-terminal kinase, act upstream of the p90rsk kinases[52-55].

Little is known about the function of RSK1 and RSK3. The *RSK1* gene is localized on chromosome 3, and its expression is restricted to hematopoietic and HeLa cells. *RSK3* has been mapped to chromosome 6 and contains a unique N-terminal sequence[44,45]. *RSK2*, which has been localized to Xp22, is mutated in Coffin-Lowry syndrome (CLS; MIM 303600)[56,57], a disorder characterized by mental retardation, characteristic facies, and tapering fingers[58-60]. Recently, a mutation in *RSK2* was found in a family with MRX[61]. In keeping with its involvement in mental retardation is the demonstration that two known substrates of RSK2 have been implicated in brain function: L1CAM, which is mutated in several syndromic forms of MRX[62], and c-AMP response element binding protein, which is important for long-term memory formation[63-65]. These observations suggest that *RSK4* is an attractive candidate gene for MRX.

The products of two of the known MRX genes interact with the Rho-subfamily of small GTPases: *OPHN1*[9] and *PAK3*[10]. Rho proteins are involved in the organization of the actin cytoskeleton and in the generation of cell extensions in several cell types, including

neuronal cells[66-68]. *OPHN1* encodes a RHO-GTPase-activating protein, which shows GAP activity for Rho, Rac, and Cdc42 *in vitro*[9]. *PAK3* is a member of the p21-activating kinase genes, which link RHO-GTPases to the MAP kinase signaling pathway[69]. RSKs can be activated by this signaling cascade[70], suggesting that RSK2 and probably RSK4 play a role in neuronal development and synaptic function[71] (Figure 6).

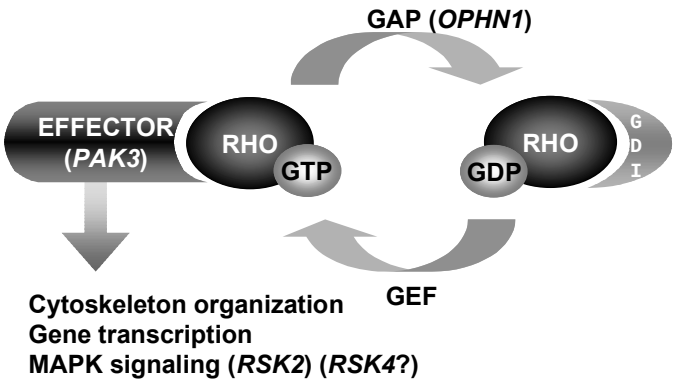

Figure 6. *Model for the role of RHO-GTPases in neuronal processes. RHO-GTPases function as molecular switches that cycle between their active (GTP-bound) and inactive (GDP-bound) states. Guanine-nucleotide dissociation inhibitors (GDIs) stabilize the inactive state. The ratio of the two forms is regulated by guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). OPHN1 is such a RHOGAP protein, and PAK3 is an effector of RHO GTPases. Downstream cascades of these effectors include the MAP kinase signaling pathway in which RSK proteins play an important role. Mutations in OPHN1, PAK3, and RSK2 are involved in MRX[9,10,60]. (Adapted from Antonarakis and Van Aelst[71])*

Despite the fact that *RSK4* is a positional and functional candidate gene for MRX, we failed to detect causative mutations in 200 unrelated male MRX patients. The fact that no mutations have been found, together with the partial deletion of the gene in patient VS, may suggest that *RSK4* is not the gene underlying MRX in Xq21. On the other hand, the absence of other expressed sequences in this region (Yntema *et al.*, unpublished observations) and the general paucity of genes in Xq21[24] argue in favor of *RSK4* being the Xq21 MRX gene. The normal mental status of patient VS can be explained if VS is considered to be an example of nonpenetrance. This is not unprecedented for Xq21 deletions, as two patients, NP and RVD, with deletions encompassing *POU3F4* and *REP-1*, who did not show the characteristics of DFN3 or CHM, respectively, have been described[12]. An explanation for the lack of phenotypic features in these patients is that the absence of protein can be compensated for by the expression of homologous genes with a similar function. In the case of patient VS, it can be envisaged that the role of RSK4 can be taken over by one of the other RSK proteins. Functional studies in the near future must attempt to gain more insight into this hypothesis.

In all MRX genes described at present, mutations have been found in less than 1% of the patients tested. If *RSK4* is indeed causative in MRX, its contribution to mental retardation might even be lower than that of the other reported MRX genes, given the fact that no mutations were found in 200 patients tested. Therefore, more patients will be tested in the near future. These studies will concentrate on families in whom the mental retardation defect is linked to Xq21, such as MRX26[72] and FG syndrome[73]. Additionally, a group of sporadic patients with Coffin-Lowry syndrome, in whom no mutation in the *RSK2* gene was found[55], will be included in these studies.

REFERENCES

1. Lubs H, Chiurazzi P, Arena J, Schwartz C, Tranebjaerg L, and Neri G (1999) XLMR genes: update 1998. *Am J Med Genet* **83**: 237-247.
2. Kerr B, Turner G, Mulley J, Gedeon A, and Partington M (1991) Non-specific X-linked mental retardation. *J Med Genet* **28**: 378-382.
3. Herbst DS, and Miller JR (1980) Nonspecific X-linked mental retardation II: the frequency in British Columbia. *Am J Med Genet* **7**: 461-469.
4. Gedeon AK, Donnelly AJ, Mulley JC, Kerr B, and Turner G (1996) How many X-linked genes for non-specific mental retardation (MRX) are there? *Am J Med Genet* **64**: 158-162.
5. Gecz J, Gedeon AK, Sutherland GR, and Mulley JC (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation. *Nat Genet* **13**: 105-108.
6. Gu Y, Shen Y, Gibbs RA, and Nelson DL (1996) Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island. *Nat Genet* **13**: 109-113.
7. Bienvenu T, des Portes V, Saint Martin A, McDonell N, Billuart P, Carrié A, Vinet M-C, Couvert P, Toniolo D, Ropers H-H, Moraine C, van Bokhoven H, Fryns J-P, Kahn A, Beldjord C, and Chelly J (1998) Non-specific X-linked semidominant mental retardation by mutations in a Rab GDP-dissociation inhibitor. *Hum Mol Genet* **7**: 1311-1315.
8. D'Adamo P, Menegon A, Lo Nigro C, Grasso M, Gulisano M, Tamanini F, Bienvenu T, Gedeon AK, Oostra B, Wu S-K, Tandon A, Valtorta F, Balch WE, Chelly J, and Toniolo D (1998) Mutations in *GDI1* are responsible for X-linked non-specific mental retardation. *Nat Genet* **19-6**: 134-139.
9. Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC, Zemni R, Roest Crollius H, Carrié A, Fauchereau F, Cherry M, Briault S, Hamel B, Fryns J-P, Beldjord C, Kahn A, Moraine C, and Chelly J (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**: 923-926.
10. Allen KM, Gleeson JG, Bagrodia S, Partington MW, MacMillan JC, Cerione RA, Mulley JC, and Walsh CA (1998) *PAK3* mutation in nonsyndromic X-linked mental retardation. *Nat Genet* **20**: 25-30.
11. Bach I, Robinson D, Thomas N, Ropers H-H, and CremersFPM (1992) Physical fine mapping of genes underlying X-linked deafness and non fra(X)-X-linked mental retardation at Xq21. *Hum Genet* **89**: 620-624.
12. Cremers FPM, van de Pol DJR, Diergaarde PJ, Wieringa B, Nussbaum RL, Schwartz M, and Ropers H-H (1989) Physical fine mapping of the choroideremia locus using Xq21 deletions associated with complex syndromes. *Genomics* **4**: 41-46.
13. May M, Colleaux L, Murgia A, Aylsworth A, Nussbaum R, Fontes M, and Schwartz C (1995) Molecular analysis of four males with mental retardation and deletions of Xq21 places the putative MR region in Xq21.1 between DXS233 and CHM. *Hum Mol Genet* **4**: 1465-1466.
14. Bach I, Brunner HG, Beighton P, Ruvalcaba RHA, Reardon W, Pembrey ME, van der Velde-Visser SD, Bruns GAP, Cremers CWRJ, Cremers FPM, and Ropers H-H (1992) Microdeletions in patients with gusher-associated, X-linked mixed deafness (DFN3). *Am J Hum Genet* **50**: 38-44.
15. Cremers FPM, Sankila E-M, Brunsmann F, Jay M, Jay B, Wright A, Pinckers AJLG, Schwartz M, van de Pol DJR, Wieringa B, de la Chapelle A, Pawlowitzki IH, and Ropers H-H (1990) Deletions in patients with classical choroideremia vary in size from 45 kb to several megabases. *Am J Hum Genet* **47**: 622-628.
16. de Kok YJM, Vossenaar ER, Cremers CWRJ, Dahl N, Laporte J, Hu LJ, Lacombe D, Fischel-Ghodsian N, Friedman RA, Parnes LS, Thorpe P, Bitner-Glindzicz M, Pander H-J, Heilbronner H, Graveline J, den Dunnen JT, Brunner HG, Ropers H-H, and Cremers FPM (1996) Identification of a hot spot for microdeletions in patients with X-linked deafness type 3 (DFN3) 900 kb proximal to the DFN3 gene *POU3F4*. *Hum Mol Genet* **5**: 1229-1235.
17. Huber I, Bitner-Glindzicz M, de Kok YJM, van der Maarel SM, Ishikawa-Brush Y, Monaco AP, Robinson D, Malcolm S, Pembrey ME, Brunner HG, Cremers FPM, and Ropers H-H (1994) X-linked mixed deafness (DFN3): cloning and characterization of the critical region allows the identification of novel microdeletions. *Hum Mol Genet* **3**: 1151-1154.
18. Sankila E-M, Bruns GAP, Schwartz M, Nikoskelainen E, Niebuhr E, Hodgson SV, Wright AF, and de la Chapelle A (1990) DXS26 (HU16) is located in Xq21.1. *Hum Genet* **85**: 117-120.
19. van Bokhoven H, van den Hurk JAJM, Bogerd L, Philippe C, Gilgenkrantz S, de Jong P, Ropers H-H, and Cremers FPM (1994) Cloning and characterization of the human choroideremia gene. *Hum Mol Genet* **3**: 1041-1046.
20. van der Maarel SM, Scholten IHJM, Maat-Kievit JA, Huber I, de Kok YJM, de Wijs I, van de Pol TJR, van Bokhoven H, den Dunnen JT, van Ommen GJB, Philippe C, Monaco AP, Smeets HJM, Ropers H-H, and Cremers FPM (1995) Yeast artificial chromosome cloning of the Xq13.3-q21.31 region and fine mapping of a deletion associated with choroideremia and nonspecific mental retardation. *Eur J Hum Genet* **3**: 207-218.
21. Cremers FPM, van de Pol DJR, van Kerkhoff LPM, Wieringa B, and Ropers H-H (1990) Cloning of a gene that is rearranged in patients with choroideremia. *Nature* **347**: 674-677.
22. de Kok YJM, van der Maarel SM, Bitner-Glindzicz M, Huber I, Monaco AP, Malcolm S, Pembrey ME, Ropers H-H, and Cremers FPM (1995) Association between X-linked mixed deafness and mutations in the POU domain gene *POU3F4*. *Science* **267**: 685-688.
23. van Bokhoven H, Schwartz M, Andréasson S, van den Hurk JAJM, Bogerd L, Jay M, Rüther K, Jay B, Pawlowitzki IH, Sankila E-M, Wright A, Ropers H-H, Rosenberg T, and Cremers FPM (1994) Mutation spectrum in the CHM gene of Danish and Swedish choroideremia patients. *Hum Mol Genet* **3**: 1047-1051.
24. Philippe C, Arnould C, Sloan F, van Bokhoven H, van der Velde-Visser SD, Chery M, Ropers H-H, Gilgenkrantz S, Monaco AP, and Cremers FPM (1995) A high resolution interval map of the q21 region of the human X chromosome. *Genomics* **27**: 539-543.
25. Miller SA, Dykes DD, and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**: 1215-1215.
26. Hu L-J, Blumenfeld-Heyberger S, Hanauer A, Weissenbach J, and Mandel J-L (1994) Non-specific X-linked mental retardation: linkage analysis in MRX2 and MRX4 families revisited. *Am J Med Genet* **51**: 569-574.
27. Kerr B, Gedeon A, Mulley J, and Turner G (1992) Localization of non-specific X linked mental retardation genes. *Am J Med Genet* **43**: 392-401.
28. Lazzarini A, Stenroos ES, Lehner T, McKoy V, Gold B, McCormack MK, Reid CS, Ott J, and Johnson WG (1995) Short tandem repeat polymorphism linkage studies in a new family with X-linked mental retardation (MRX20). *Am J Med Genet* **57**: 552-557.
29. Hamel BCJ, Smits APT, van den Helm B, Smeets DFCM, Knoers NVAM, van Roosmalen T, Thoonen GHJ, Assman-Hulsmans CFCH, Ropers H-H, Mariman ECM, and Kremer H (1999) Four families (MRX43, MRX44, MRX45, MRX52) with nonspecific X-linked mental retardation: clinical and psychometric data and results of linkage analysis. *Am J Med Genet* **85**: 290-304.
30. Yntema HG, van den Helm B, Knoers NVAM, Smits APT, van Roosmalen T, Smeets DFCM, Mariman ECM, van dBI, van Bokhoven H, Ropers H-H, Kremer H, and Hamel BCJ (1999) X-linked mental retardation: evidence for a recent mutation in a five-generation family (MRX65) linked to the pericentromeric region. *Am J Med Genet* **85**: 305-308.
31. Wilson M, Mulley J, Gedeon A, Robinson H, and Turner G (1991) New X-linked syndrome of mental retardation, gynecomastia, and obesity is linked to DXS255. *Am J Med Genet* **40**: 406-413.

32. Kremer H, Hamel BCJ, van den Helm B, Arts WFM, de Wijs IJ, Sistermans EA, Ropers HH, and Mariman ECM (1996) Localization of the gene (or genes) for a syndrome with X-linked mental retardation, ataxia, weakness, hearing impairment, loss of vision and a fatal course in early childhood. *Hum Genet* **98**: 513-517.
33. Hamel BCJ, Mariman ECM, van Beersum SEC, Schoonbrood-Lenssen AMJ, and Ropers H-H (1994) Mental retardation, congenital heart defect, cleft palate, short stature, and facial anomalies: a new X-linked multiple congenital anomalies/mental retardation syndrome: clinical description and molecular studies. *Am J Med Genet* **51**: 591-597.
34. Cotter FE, Hampton GM, Nasipuri S, Bodmer WF, and Young BD (1990) Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid. *Genomics* **7**: 257-263.
35. Datson NA, van de Vosse E, Dauwerse HG, Bout M, van Ommen GJB, and den Dunnen JT (1996) Scanning for genes in large genomic regions: cosmid-based exon trapping of multiple exons in a single product. *Nucleic Acids Res* **24**: 1105-1111.
36. Feinberg AP and Vogelstein B (1984) "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity". Addendum. *Anal Biochem* **137**: 266-267.
37. Orita M, Suzuki Y, Sekiya T, and Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5**: 874-879.
38. van den Hurk JAJM, van de Pol TJR, Molloy CM, Brunsmann F, Rüther K, Zrenner E, Pinckers AJLG, Pawlowitzki IH, Bleeker-Wagemakers EM, Wieringa B, Ropers H-H, and Cremers FPM (1992) Detection and characterization of point mutations in the choroideremia candidate gene by PCR-SSCP analysis and direct DNA sequencing. *Am J Hum Genet* **50**: 1195-1202.
39. Colleaux, L., May, M., Belougne, J., Lepaslier, D., Schwartz, C., and Fontes, M. (1996). Localisation of two candidate genes for mental retardation using a YAC physical map of the Xq21.1-21.2 subbands. *J. Med. Genet.* **33:** 235-357.
40. Pearson WR and Lipman DJ (1988) Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**: 2444-2448.
41. Shapiro MB and Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res* **15**: 7155-7174.
42. Antonarakis SE, and The Nomenclature Working Group (1998) Recommendations for a nomenclature system for human gene mutations. *Hum Mutat* **11**: 1-3.
43. Alcorta DA, Crews CM, Sweet LJ, Bankston L, Jones SW, and Erikson RL (1989) Sequence and expression of chicken and mouse rsk: homologs of Xenopus laevis ribosomal S6 kinase. *Mol Cell Biol* **9**: 3850-3859.
44. Moller DE, Xia CH, Tang W, Zhu AX, and Jakubowski M (1994) Human rsk isoforms: cloning and characterization of tissue-specific expression. *Am J Physiol* **266**: C351-C359.
45. Zhao Y, Bjorbaek C, Weremowicz S, Morton CC, and Moller DE (1995) RSK3 encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. *Mol Cell Biol* **15**: 4353-4363.
46. Jones SW, Erikson E, Blenis J, Maller JL, and Erikson RL (1988) A *Xenopus* ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases. *Proc Natl Acad Sci USA* **85**: 3377-3381.
47. Chen RH, and Blenis J (1990) Identification of *Xenopus* S6 protein kinase homologs ($pp90^{rsk}$) in somatic cells: phosphorylation and activation during initiation of cell proliferation. *Mol Cell Biol* **10**: 3204-3215.
48. Chen RH, Chung J, and Blenis J (1991) Regulation of $pp90^{rsk}$ phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cyclic AMP-mediated signal transduction. *Mol Cell Biol* **11**: 1861-1867.
49. Chung J, Chen RH, and Blenis J (1991) Coordinate regulation of $pp90^{rsk}$ and a distinct protein- serine/threonine kinase activity that phosphorylates recombinant $pp90^{rsk}$ in vitro. *Mol Cell Biol* **11**: 1868-1874.
50. Cobb MH, Boulton TG, and Robbins DJ (1991) Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul* **2**: 965-978.
51. Sturgill TW and Wu J (1991) Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim Biophys Acta* **1092**: 350-357.
52. Sutherland C, Campbell DG, and Cohen P (1993) Identification of insulin-stimulated protein kinase-1 as the rabbit equivalent of rskmo-2. Identification of two threonines phosphorylated during activation by mitogen-activated protein kinase. *Eur J Biochem* **212**: 581-588.
53. Chen RH, Sarnecki C, and Blenis J (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol Cell Biol* **12**: 915-927.
54. Grove JR, Price DJ, Banerjee P, Balasubramanyam A, Ahmad MF, and Avruch J (1993) Regulation of an epitope-tagged recombinant Rsk-1 S6 kinase by phorbol ester and erk/MAP kinase. *Biochemistry* **32**: 7727-7738.
55. Moxham CM, Tabrizchi A, Davis RJ, and Malbon CC (1996) Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo. *J Biol Chem* **271**: 30765-30773.
56. Jacquot S, Merienne K, De Cesare D, Pannetier S, Mandel JL, Sassone-Corsi P, and Hanauer A (1998) Mutation analysis of the RSK2 gene in Coffin-Lowry patients: extensive allelic heterogeneity and a high rate of de novo mutations. *Am J Hum Genet* **63**: 1631-1640.
57. Trivier E, De Cesare D, Jacquot S, Pannetier S, Zackai E, Young I, Mandel J-L, Sassone-Corsi P, and Hanauer A (1996) Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. *Nature* **384**: 567-570.
58. Coffin GS, Siris E, and Wegienka LC (1966) Mental retardation with osteocartilagenous anomalies. *Am J Dis Child* **112**: 205-213.
59. Lowry B, Miller JR, and Fraser FC (1971) A new dominant gene mental retardation syndrome. Association with small stature, tapering fingers, characteristic facies, and possible hydrocephalus. *Am J Dis Child* **121**: 496-500.
60. Temtamy SA, Miller JD, and Hussels-Maumenee I (1975) The Coffin-Lowry syndrome: an inherited faciodigital mental retardation syndrome. *J Pediatr* **86**: 724-731.
61. Merienne K, Jacquot S, Pannetier S, Zeniou M, Bankier A, Gecz J, Mandel J-L, Mulley J, Sassone-Corsi P, and Hanauer A (1999) A missense mutation in *RPS6KA3* (*RSK2*) responsible for non-specific mental retardation. *Nat Genet* **22**: 13-14.
62. Fransen E, Van Camp G, Vits L, and Willems PJ (1997) L1-associated diseases: clinical geneticists divide, molecular geneticists unite. *Hum Mol Genet* **6**: 1625-1632.
63. Carew TJ (1996) Molecular enhancement of memory formation. *Neuron* **16**: 5-8.
64. Pende M, Fisher TL, Simpson PB, Russell JT, Blenis J, and Gallo V (1997) Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway. *J Neurosci* **17**: 1291-1301.
65. Xing J, Ginty DD, and Greenberg ME (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**: 959-963.
66. Luo L, Jan LY, and Jan YN (1997) Rho family GTP-binding proteins in growth cone signalling. *Curr Opin Neurobiol* **7**: 81-86.
67. Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, and Jan YN (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**: 837-840.
68. Threadgill R, Bobb K, and Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron* **19**: 625-634.
69. Sells MA and Chernoff J (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol* **7**: 162-167.

70. Seger R and Krebs EG (1995) The MAPK signaling cascade. *FASEB J* **9**: 726-735.
71. Antonarakis SE, and Van Aelst L (1998) Mind the GAP, Rho, Rab and GDI. *Nat Genet* **19**: 106-108.
72. Robledo R, Melis P, Laficara F, Marchi J, Rinaldi A, Siniscalco M, and Filippi G (1996) Further linkage evidence for localization of mutational sites for nonsyndromic types of X-linked mental retardation at the pericentromeric region. *Am J Med Genet* **64**: 107-112.
73. Briault S, Hill R, Shrimpton A, Zhu D, Till M, Ronce N, Margaritte-Jeannin P, Baraitser M, Middleton-Price H, Malcolm S, Thompson E, Hoo J, Wilson G, Romano C, Guichet A, Pembrey M, Fontes M, Poustka A, and Moraine C (1997) A gene for FG syndrome maps in the Xq12-q21.31 region. *Am J Med Genet* **73**: 87-90.

Appendix 4

Mutations in *ARHGEF6*, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation

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Published in: *Nat Genet* **26:** 247-250 (2000)

ABSTRACT

X-linked forms of mental retardation (XLMR) include a variety of different disorders and may account for up to 25% of all inherited cases of mental retardation[1]. So far, seven X-chromosomal genes mutated in nonspecific mental retardation (MRX) have been identified: *FMR2*, *GDI1*, *RPS6KA3*, *IL1RAPL*, *TM4SF2*, *OPHN1* and *PAK3*[2-9]. The products of the latter two have been implicated in regulation of neural plasticity by controlling the activity of small GTPases of the Rho family. Here we report the identification of a new MRX gene, *ARHGEF6* (also known as *αPIX* or *Cool-2*), encoding a protein with homology to guanine nucleotide exchange factors for Rho GTPases (Rho GEF). Molecular analysis of a reciprocal X/21 translocation in a male with mental retardation showed that this gene in Xq26 was disrupted by the rearrangement. Mutation screening of 119 patients with nonspecific mental retardation revealed a mutation in the first intron of *ARHGEF6* (IVS1-11T→C) in all affected males in a large Dutch family[10]. The mutation resulted in preferential skipping of exon 2, predicting a protein lacking 28 amino acids. *ARHGEF6* is the eighth MRX gene identified so far and the third such gene to encode a protein that interacts with Rho GTPases.

RESULTS AND DISCUSSION

The patient (FCA) we studied shows severe mental retardation, mild dysmorphic features and sensorineural hearing loss. He carries an apparently balanced X;21 translocation with breakpoints in Xq26 and 21p11. His mother (VCA) is unaffected and carries the same translocation[11]. To clone the breakpoint region, we performed fluorescence in situ hybridization (FISH) with YAC, PAC and cosmid clones from Xq26 and screened for clones spanning the breakpoint. No single clone was found that contained genetic material located on both sides of the breakpoint. Therefore, we subcloned PAC H211017 and cosmid B1220 mapped nearest but still proximal and distal to the breakpoint, respectively. Clones were end-sequenced and assembled into a contig. DNA sequences of both ends of a 5.1-kb DNA fragment showed partial homology to cDNA KIAA0006[12], suggesting the presence of two exons. The KIAA0006 cDNA sequence deposited in the database contains only part of the coding sequence, as no ATG is present at the beginning of the ORF. Therefore, we performed 5'-RACE experiments and identified an additional 1,213 bp at the 5' end. The complete cDNA sequence (6,017 bp) encodes a protein of 776 amino acids with a predicted molecular weight of 87 kD. The putative initiation codon at nt 1,207 conforms to a translation-initiation consensus[13], and is preceded by several stop codons in all reading frames (data not shown). Database searches revealed that the predicted product of KIAA0006 is a Rho GEF protein[14] (ARHGEF6; Figure 1). Rho GEFs belong to a family of cytoplasmic proteins that activate the Ras-like family of Rho proteins by exchanging bound GDP for GTP[15,16]. The ARHGEF6 polypeptide contains several evolutionarily conserved regions frequently found in other

Rho GEF family members (Figure 1): for example, an SH3 domain, a Dbl homology (DH) domain[17] and a pleckstrin homology (PH) domain[18,19]. The amino-terminal calponin homology (CH) domain[20] is unique to ARHGEF6. We determined the genomic structure of *ARHGEF6* and identified 22 exons (Figure 1). Previous studies have shown that *ARHGEF6* is expressed ubiquitously[12,14]. Using a commercially available RNA array from multiple adult human tissues, we found no significant differences in the expression pattern of different parts of the brain (data not shown).

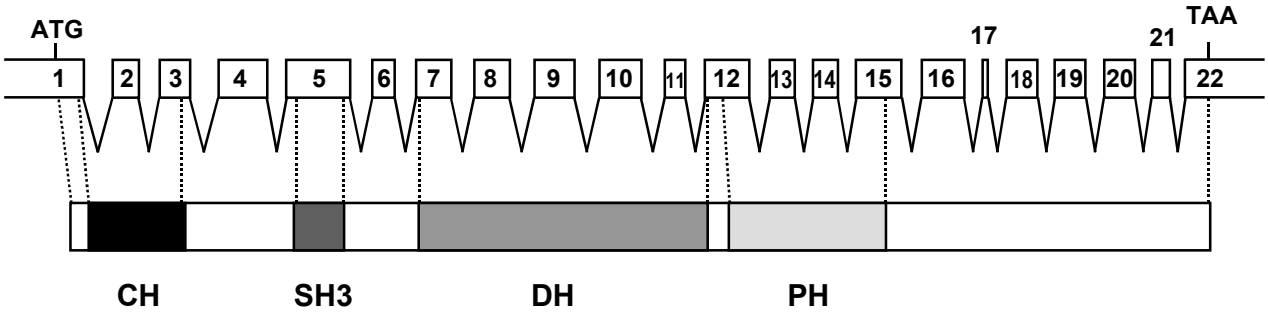

***Figure 1**. Predicted genomic structure of ARHGEF6 and domain structure of the corresponding protein. Numbered boxes indicate exons. The ATG start codon and TAA stop codon are shown. Schematic organization of the identified domains in the ARHGEF6 protein is shown below: CH, calponin homology domain; SH3, Src homology 3 domain; DH, Dbl homology domain; PH, pleckstrin homology domain.*

To further characterize the translocation breakpoint, we performed serial RT–PCR on RNA isolated from skin fibroblasts of the patient, his mother and a control individual. We obtained products corresponding to exons 8 to 10 in all cases (Figure 2A), but amplification of exons 8–11 and exons 8–12 was unsuccessful in the patient, suggesting that the translocation breakpoint was located between exons 10 and 11 (Figure 2A). We detected wild-type *ARHGEF6* mRNA in the patient's mother, who carries the same chromosomal rearrangement as her son. This is unusual, because in most females with a balanced X/autosome translocation the normal X chromosome is preferentially inactivated. This finding, however, is consistent with the mother's normal phenotype. To investigate whether *ARHGEF6* escapes X inactivation, we performed RT–PCR using RNA from hamster somatic cell hybrids that contained either an active (Xa) or an inactive (Xi) human X chromosome. We obtained PCR products only from the hybrids with Xa (data not shown), indicating that *ARHGEF6* does not escape X inactivation.

The chromosomal breakpoint of patient FCA was further characterized by genomic walking. Two different junction-containing DNA fragments comprising the der(X) and der(21) breakpoints were amplified and sequenced (Figure 2B–D). The der(21) breakpoint is at nt 650 in intron 10, whereas the breakpoint on der(X) is before nt 660 in the same *ARHGEF6* intron. DNA sequences of both junction fragments adjacent to *ARHGEF6* show homology to β-satellite DNA (Figure 2C), suggesting that the translocation is reciprocal but molecularly unbalanced, with a 10-bp deletion on the X chromosome and a 9-bp deletion in the β-satellite DNA on chromosome 21 (data not shown). We found two short motifs of sequence identity (TGCAATGA and TGATA (Figure 2C)) on both the X chromosome and chromosome 21.

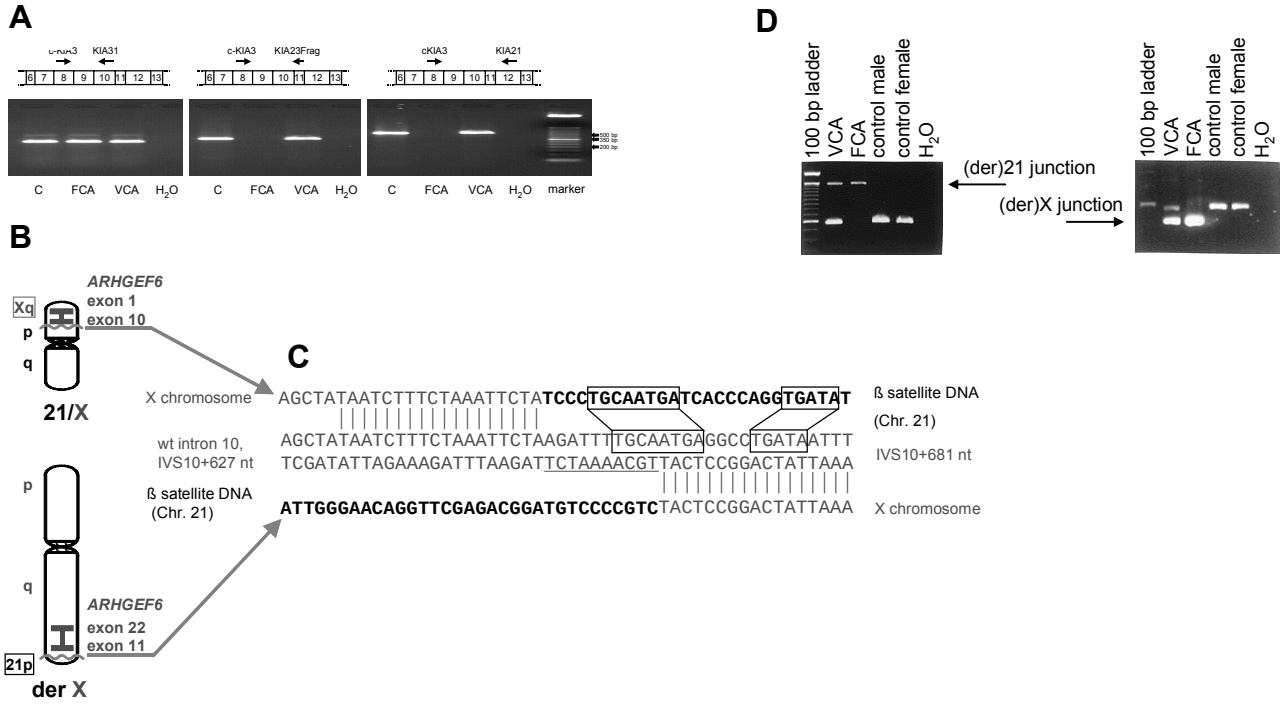

Figure 2. *Mapping the translocation breakpoint by the analysis of cDNA and genomic DNA fragments from patient FCA.* ***A:*** *RT–PCR amplification of different ARHGEF6 exons from the RNA of the patient (FCA), his mother (VCA) and a control person (c). Boxes indicate exons of ARHGEF6.* ***B:*** *Schematic representation of the rearranged chromosome 21 and the derivative X chromosome. The translocated part of the X chromosome (Xq26–qter) harbors ARHGEF6 exons 1–10. Exons 11–22 are on the derivative X chromosome oriented from telomere to centromere.* ***C:*** *DNA sequence of the fragments and the corresponding wild-type DNA segment of intron 10 of ARHGEF6. Top, the DNA sequence of the breakpoint on the rearranged chromosome 21 compared with that of wild-type intron 10 (middle line). Bottom, the sequence of the breakpoint on the derivative X chromosome. Vertical lines indicate identical nucleotides of the junction fragments and intron 10. -satellite DNA of chromosome 21 is in bold. Homologous DNA sequences of -satellite DNA and ARHGEF6 intron 10 are boxed. The 10 bp of intron 10 deleted by the rearrangement are underlined.* ***D:*** *Amplification of translocation junction fragments by chromosome X- and 21-specific PCR primers. A novel 1.5-kb amplicon (marked by an arrow) was generated for the der(21) chromosome (left) and a 510-bp amplicon for the der(X) chromosome (right) in patient FCA and his mother VCA. These products were not obtained in controls. A control fragment of 580 bp containing the breakpoint region in intron 10 of ARHGEF6 on the wild-type allele was co-amplified in the same PCR reactions.*

Subsequently, we screened 119 unrelated male patients with nonspecific mental retardation for mutations in *ARHGEF6*. We identified a sequence variant in intron 1, IVS1-11TC (Figure 3A,B), in a patient (III-20 in Fig. 4A) from a large family in which the disease locus (MRX46) was previously assigned to Xq25–q26[10]. This base change has a marginal effect on the predicted splicing efficiency (79% versus 77% in the mutant[21]), but was not detected on 170 control chromosomes. We showed that IVS1-11TC segregated with the disease phenotype in the family (data not shown). To determine the effect of this sequence variant on splicing, we performed RT–PCR amplifying exons 1–4 of *ARHGEF6* cDNA from 4 affected males (Figure 4A) and obtained products of 2 different sizes (Figure 4B). We cloned and sequenced these products, and found that the larger amplicon corresponded to the wild-type fragment, whereas in the smaller amplicon exon 1 was spliced to exon 3. Thus, all mentally retarded males in MRX46 exhibited enhanced skipping of exon 2. In this nonquantitative analysis, we did not see skipping of exon 2 in 2 carrier females or in an unaffected male (Figure 4B). Similarly, no skipping of exon 2 was

found by qualitative RT–PCR analysis of 100 unaffected controls (data not shown). We estimated the relative amount of the wild-type and truncated RNA from lymphoblastoid cell lines by a semi-quantitative RT–PCR, and found it to be approximately 1:3 in patients III-16, III-20 and IV-1 (with moderate mental retardation), and approximately 1:5 for patient III-26 (with profound mental retardation; Figure 4C). In this assay, we also detected small amounts of the product without exon 2 in obligate carriers and the unaffected male (Figure 4C), suggesting that skipping of exon 2 is a rare, but normal, event in lymphoblastoid cell lines. In 52 control individuals, the amount of the minor cDNA fragment was found to be 3–10% of the total product (data not shown).

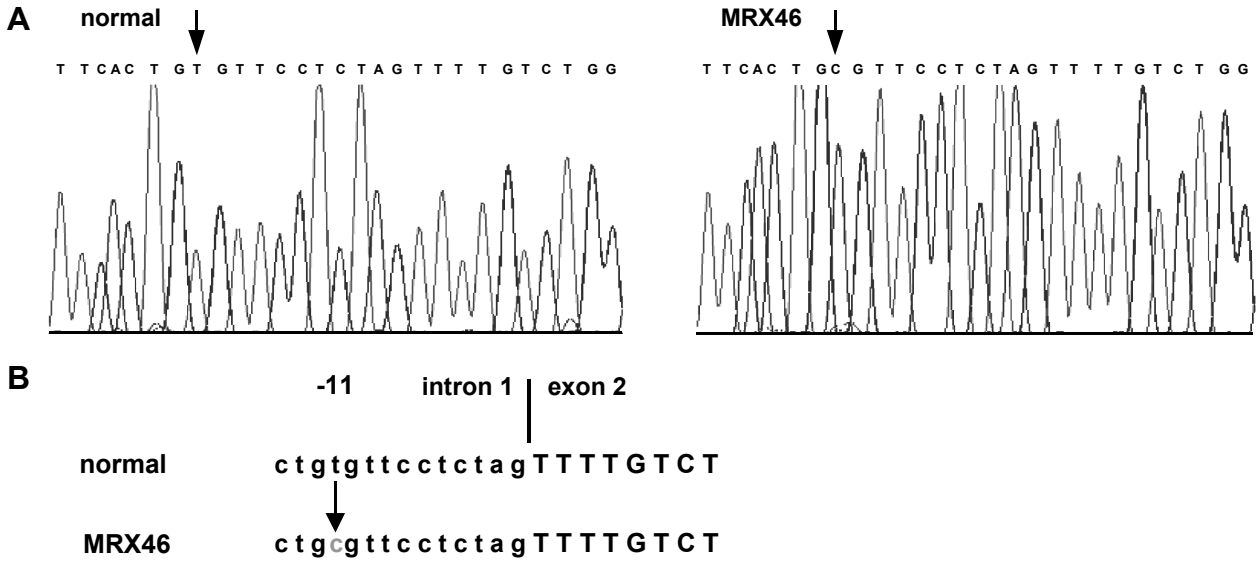

Figure 3. *Mutation analysis of ARHGEF6 in MRX46.* ***A:*** *PCR products of amplicon 2 were sequenced and the intronic mutation IVS1-11TC was detected in patients of family MRX46. Control female has a T at position -11 in the splice acceptor site of intron 1 (arrow), whereas an affected male of family MRX46 has a C at this position.* ***B:*** *Wild-type DNA sequence of the splice acceptor site of intron 1 and exon 2 is shown at the top, whereas the sequence below presents the intronic mutation of MRX46.*

SSCP analysis revealed that five women from MRX46 were heterozygous for the IVS1-11TC mutation (Fig. 4A). In two carriers (III-3 and IV-3), we found only a small amount of mRNA lacking exon 2 by quantitative RT–PCR (Fig. 4C) that might originate from the wild-type allele. Indeed, all carrier females presented a skewed pattern of X inactivation[22], whereas three non-carriers (III-10, III-19 and IV-7) showed a random pattern (Fig. 4D, and data not shown). Genotyping of DNA polymorphisms showed that in all carrier females the X chromosome with the intron 1 mutation in *ARHGEF6* was inactivated (data not shown).

ARHGEF6 is the third MRX gene, in addition to *OPHN1* and *PAK3*, whose protein is involved in the Rho GTPase cycle, which mediates organization of the cytoskeleton, cell shape and motility[23,24]. As it has already been shown that Rho GTPases are involved in axonal outgrowth, as well as in control of shape and size of dendrites[25], mutations of *ARHGEF6* may disturb signal transduction pathways involved in cell migration and axonal outgrowth. The phenotypes of the patients carrying mutations in *ARHGEF6* were

different, underscoring a complex genetic heterogeneity in MRX[5,26]. The molecular consequences of the gene disruption in patient FCA and preferential skipping of exon 2 in family MRX46 are unknown at the level of cell physiology. We speculate that mental retardation in family MRX46 might be due to the switch in the ratio between the two ARHGEF6 isotypes, which has already been described in early onset familial adenomatous polyposis[27].

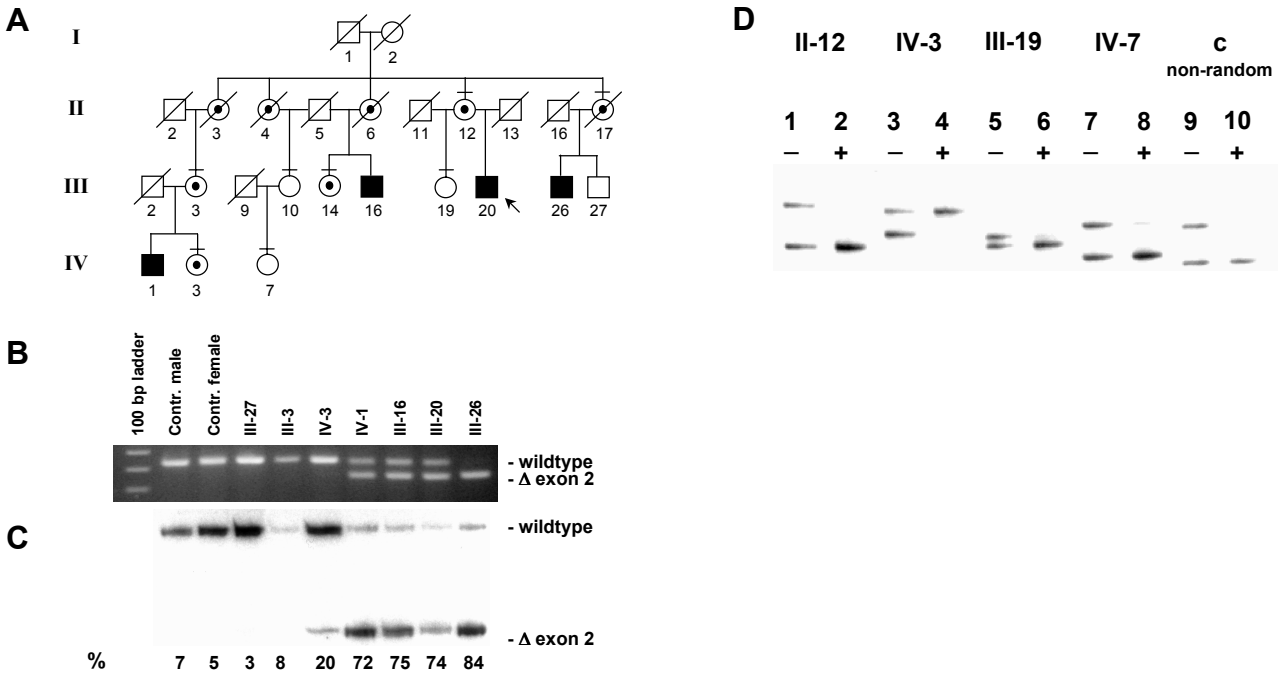

Figure 4. *Analysis of the splice mutation and X-inactivation study in family MRX46.* ***A:*** *Simplified pedigree of family MRX46 (original in [10]). Pedigree numbers are the same as given in the original. Carrier women are indicated by a dot and, if determined by DNA analysis, by a dash. The index case is indicated by an arrow.* ***B:*** *Nonquantitative amplification of ARHGEF6 exons 1–4 by RT–PCR and analysis of products on a 1.5% agarose gel. Lane 1, 100-bp ladder; lane 2, male control; lane 3, female control; lane 4, non-affected male (III-27); lane 5, carrier female (III-3); lane 6, carrier female (IV-3); lane 7, affected male (IV-1); lane 8, affected male (III-16, III-20, III-26). All affected males show an additional, smaller amplicon.* ***C:*** *Semi-quantitative RT–PCR of ARHGEF6 exons 1–4 analyzed by denaturing polyacrylamide gel. The top band contains wild-type ARHGEF6 exons 1–4, the bottom band lacks exon 2. The patients studied here are the same as in* ***B****. The percentage of ARHGEF6 transcript lacking exon 2 is indicated below.* ***D:*** *X-chromosome inactivation determined by amplification of a sequence polymorphism of the gene encoding the androgen receptor with or without digestion of the DNA with HpaII (indicated with + and -, respectively). Carrier females II-12 and IV-3 show nonrandom X inactivation, as only one band is obtained after HpaII digestion. In contrast, non-carriers III-19 and IV-7 show a random X inactivation. The pattern of a nonrandom X-inactivated control is shown in lanes 9 and 10.*

METHODS

YAC, PAC and cosmid clones

We obtained YAC clones from American Type Culture Collection. PACs from libraries RPCI-3 and -5 (P. de Jong and P. Ioannou) and cosmid clones from library 110 (P. de Jong) were provided by the Resource Center of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics.

Labeling of probes and FISH

Metaphase spreads were made by standard procedures. YAC, PAC and cosmid DNA were labeled with digoxigenin-11-dUTP (Roche Molecular biochemicals) by nick translation (Roche Molecular Biochemicals). We used fluorescein isothiocyanate (FITC) labeled streptavidin (1:200) for detection. Chromosomes were counterstained with propidium iodide. Slides were observed under a Zeiss Axiophot fluorescence microscope.

Mapping of the t(X;21) breakpoint

*Eco*RI fragments of cosmid LLNLc110B1220Q03 and PAC RPCIP704H211017Q3 were subcloned in pBluescript II SK and pACYC184 and sequenced from both ends using the ABI Prism Dye Terminator kit and ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). For assembly of the *Eco*RI fragments, we designed a reverse primer of each end-sequence for PCR and combined each primer with all other primers on undigested PAC DNA as template (primer sequences are available on request).

Genomic walking, PCR of junction fragments and nucleic acid hybridization

To establish the exon-intron structure of *ARHGEF6*, we used cosmid (LLNLc110B1220Q03) and PAC (RPCIP704H211017Q3 and RPCIP704I24917Q3) clones for constructing genomic walking libraries (Universal Genome Walker kit, Clontech). The exon-intron boundaries were determined through sequence comparisons between cDNA and genomic sequence. For the isolation of genomic junction fragments from patient FCA, we constructed genomic walking libraries with genomic DNA. PCR amplification of junction fragments was carried out with specific primers for the der(21) and der(X) chromosomes. A control fragment of 580 bp containing the breakpoint region in intron 10 of *ARHGEF6* on the wild-type allele was co-amplified in the same PCR reactions (primer sequences available on request).

For Southern-blot analysis, we digested DNA samples (PACs, cosmids and cloned *Eco*RI fragments) with restriction endonucleases, electrophoresed them on 0.8% agarose gels and transferred them to positively charged nylon membranes (Roche Molecular Biochemicals) according to standard procedures. Labeling, hybridization and detection were carried out using the DIG-system (Roche Molecular Biochemicals). We hybridized the Multiple Tissue Expression Array (Clontech) with the 32P-labeled KIAA0006 cDNA probe by standard methods.

RNA isolation, RACE– and RT–PCR, and semi-quantitative RT–PCR

We isolated total RNA using the High Pure RNA Isolation kit (Roche Molecular Biochemicals) and performed reverse transcription of total RNA (5 μg) by random hexanucleotide priming and Superscript II (Life Technologies) according to the manufacturer's protocol. We carried out 5'-RACE experiments using Marathon-Ready

cDNA from human fetal brain (Clontech). For this, we combined nested reverse primers from exon 1 of KIAA0006 cDNA and the 5' UTR in PCR with adaptor primers (sequences available on request). We sequenced RACE–PCR products directly or after cloning in pBluescript II SK. We studied the effect of X-inactivation on expression of *ARHGEF6* by RT–PCR on somatic cell hybrids containing an active (cell line 578) or inactive copy (cell line 789) of the human X-chromosome on a hamster background (fibroblast cell line Wshg). For RT–PCR amplification the following primers were used: GEF5UTR1 (5'–AAACCAGCGTTATCCAGGATTG–3') and KIA44 (5'–GGAGGGTTGCACATCCTTTC AGG–3'). The identity of the resulting products was verified by direct sequencing.

We used the RNAzol B procedure (CAMPRO Scientific) for RNA isolation of EBV-transformed lymphoblasts from members of the MRX46 family, and from control individuals. cDNA synthesis was performed in a 10 µl reaction containing total RNA (150 ng), random hexamer primers (50 ng; Pharmacia), dNTPs (1 mM), RNAguard (4 U; Amersham) and MMLV-RT (0.5 U; Life Technologies). Incubation was for 10 min at RT, 60 min at 37 °C, and 6 min at 99 °C. We amplified cDNA products with primers c-KIA1 (5'–GACATGGCTTATATCTTTGGGAG–3') and KIA47 (5'–CTGTCTTTGACTGCCTTTGCAG CCCTGAAAC–3'). We labeled part of the PCR product by adding 15 mCi [γ-32^P] ATP and T4 polynucleotide kinase (5 U; Biolabs) for 30 min at 37 °C, electrophoresed on 5% denaturing polyacrylamide gels and exposed on a phosphor imager.

Mutation analysis and X-inactivation assay

In collaboration with the European XLMR Consortium, we investigated 35 MRX patients from Nijmegen, 26 patients from Tours, 1 patient from Berlin and 6 patients from Leuven. X-chromosomal inheritance was established by pedigree analysis. Fifty-one mentally retarded patients, for whom X-chromosomal inheritance was not ensured, were ascertained by the Institute of Human Genetics in Hamburg. We amplified the coding exons of *ARHGEF6* from genomic DNA using specific primers (primer sequences are available on request). We visualized PCR products on agarose gels before SSCP/heteroduplex analysis[28].

The androgen receptor (AR) X-inactivation assay has been described[22]. Primers for PCR amplification were as described[29]. PCR products were analyzed as described[28].

GenBank accession number

KIAA0006 cDNA 5'-end sequence, AF207831.

REFERENCES

1. Lubs H, Chiurazzi P, Arena J, Schwartz C, Tranebjaerg L, and Neri G (1999) XLMR genes: update 1998. *Am J Med Genet* **83**: 237-247.
2. Gecz J, Gedeon AK, Sutherland GR, and Mulley JC (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation. *Nat Genet* **13**: 105-108.
3. Gu Y, Shen Y, Gibbs RA, and Nelson DL (1996) Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island. *Nat Genet* **13**: 109-113.
4. D'Adamo P, Menegon A, Lo Nigro C, Grasso M, Gulisano M, Tamanini F, Bienvenu T, Gedeon AK, Oostra B, Wu S-K, Tandon A, Valtorta F, Balch WE, Chelly J, and Toniolo D (1998) Mutations in *GDI1* are responsible for X-linked non-specific mental retardation. *Nature Genet* **19**: 134-139.
5. Merienne K, Jacquot S, Pannetier S, Zeniou M, Bankier A, Gecz J, Mandel J-L, Mulley J, Sassone-Corsi P, and Hanauer A (1999) A missense mutation in *RPS6KA3* (*RSK2*) responsible for non-specific mental retardation. *Nat Genet* **22**: 13-14.
6. Carrié A, Jun L, Bienvenu T, Vinet M-C, McDonell N, Couvert P, Zemni R, Cardona A, Van Buggenhout G, Frints S, Hamel B, Moraine C, Ropers HH, Strom T, Howell GR, Whittaker A, Ross MT, Kahn A, Fryns JP, Beldjord C, Marynen P, and Chelly J (1999) A new member of the IL-1 receptor family highly expressed in hippocampus and involved in X-linked mental retardation. *Nat Genet* **23**: 25-31.
7. Zemni R, Bienvenu T, Vinet MC, Sefiani A, Carrié A, Billuart P, McDonell N, Couvert P, Francis F, Chafey P, Fauchereau F, Friocourt G, des Portes V, Cardona A, Frints S, Meindl A, Brandau O, Ronce N, Moraine C, van Bokhoven H, Ropers HH, Sudbrak R, Kahn A, Fryns JP, Beldjord C, and Chelly J (2000) A new gene involved in X-linked mental retardation identified by analysis of an X;2 balanced translocation. *Nat Genet* **24**: 167-170.
8. Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC, Zemni R, Roest Crollius H, Carrié A, Fauchereau F, Cherry M, Briault S, Hamel B, Fryns J-P, Beldjord C, Kahn A, Moraine C, and Chelly J (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**: 923-926.
9. Allen KM, Gleeson JG, Bagrodia S, Partington MW, MacMillan JC, Cerione RA, Mulley JC, and Walsh CA (1998) *PAK3* mutation in nonsyndromic X-linked mental retardation. *Nat Genet* **20**: 25-30.
10. Yntema HG, Hamel BCJ, Smits APT, van Roosmalen T, van den Helm B, Kremer H, Ropers H-H, Smeets DFCM, and van Bokhoven H (1998) Localisation of a gene for non-specific X linked mental retardation (MRX46) to Xq25-q26. *J Med Genet* **35**: 801-805.
11. von Ballestrem CL, Boavida MG, Zuther C, Carreiro MH, David D, Gal A, and Schwinger E (1996) Jumping translocation in a phenotypically normal female. *Clin Genet* **49**: 156-159.
12. Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S, Nagase T, Seki N, Ishikawa K, and Tabata S (1994) Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA Res* **1**: 27-35.
13. Kozak M (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* **15**: 8125-8148.
14. Manser E, Loo T-H, Koh C-G, Zhao Z-S, Chen X-Q, Tan L, Tan I, Leung T, and Lim L (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell* **1**: 183-192.
15. Boguski MS and McCormick F (1993) Proteins regulating Ras and its relatives. *Nature* **366**: 643-654.
16. Bourne HR, Sanders DA, and McCormick F (1990) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349**: 117-127.
17. Hart MJ, Eva A, Zangrilli D, Aaronson SA, Evans T, Cerione RA, and Zheng Y (1994) Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *J Biol Chem* **269**: 62-65.
18. Cerione RA and Zheng Y (1996) The Dbl family of oncogenes. *Curr Opin Cell Biol* **8**: 216-222.
19. Katan M and Allen VL (1999) Modular PH and C2 domains in membrane attachment and other functions. *FEBS Lett* **452**: 36-40.
20. Castresana J and Saraste M (1995) Does Vav bind to F-actin through a CH domain? *FEBS Lett* **374**: 149-151.
21. Shapiro MB and Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res* **15**: 7155-7174.
22. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, and Belmont JW (1992) Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* **51**: 1229-1239.
23. Hall A (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**: 509-514.
24. Van Aelst L and D'Souza-Schorey C (1997) Rho GTPases and signaling networks. *Genes Dev* **11**: 2295-2322.
25. Gallo G and Letourneau PC (1998) Axon guidance: GTPases help axons reach their targets. *Curr Biol* **8**: R80-R82.
26. Trivier E, De Cesare D, Jacquot S, Pannetier S, Zackai E, Young I, Mandel J-L, Sassone-Corsi P, and Hanauer A (1996) Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. *Nature* **384**: 567-570.
27. Bala S, Sulekova Z, and Ballhausen WG (1997) Constitutive APC exon 14 skipping in early-onset familial adenomatous polyposis reveals a dramatic quantitative distortion of APC gene- specific isoforms. *Hum Mutat* **10**: 201-206.
28. Bunge S, Fuchs S, and Gal A (1996) Simple and nonisotopic methods to detect unknown gene mutations in nucleic acids. 26-39.
29. Weeks DE, Nygaard TG, Neystat M, Harby LD, and Wilhelmsen KC (1995) A high-resolution genetic linkage map of the pericentromeric region of the human X chromosome. *Genomics* **26**: 39-46.

Appendix 5

The expanding phenotype of *XNP* mutations: mild mental retardation with or without childhood hypotonia

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Submitted for publication

ABSTRACT

Mutations in the *XNP* gene have been reported in ATR-X syndrome and other severe X-linked mental retardation conditions with facial dysmorphisms. Here, we describe a missense mutation in exon 18 in a family with borderline to moderate mental retardation. Like other disorders associated with an XNP mutation, skewed X-inactivation was found in all carrier females in this family. Only retrospective examination revealed childhood facial hypotonia and HbH inclusions in some of the affected males. These results expand the spectrum of clinical phenotypes known to be due to mutations in the *XNP* gene, and indicate that *XNP* mutation analysis should not be restricted to patients with severe mental retardation and characteristic facial features.

INTRODUCTION

Mutations in the *XNP* gene in Xq13.3 have first been reported in alpha thalassemia/mental retardation syndrome (ATR-X; MIM 301040), which is characterized by severe mental retardation, α-thalassemia, characteristic facial dysmorphisms, microcephaly and urogenital malformations[1]. Subsequent mutations have been reported in families affected with Juberg-Marsidi syndrome (MIM 309590)[2], Carpenter-Waziri syndrome[3], severe mental retardation with spastic paraplegia[4], Holmes-Gang syndrome[5] and Smith-Fineman-Myers syndrome (MIM 309580)[6]. All these syndromes have severe mental retardation and facial dysmorphisms. Only recently, an XNP null mutation has been reported in a family in which two patients showed moderate to profound MR and had the typical characteristic features of ATR-X syndrome, whereas two others had mild MR and epilepsy but did not have the characteristic facial dysmorphisms[7].

Lossi *et al*.[4] described that the bias in X inactivation, together with the severe MR, are the only consistent findings in all patients, and that a first selection can be based on these characteristics. About half of the mutations found in ATR-X syndrome patients are located within exons 7, 8 and 9, and sequencing of this hotspot has proven to be extremely efficient in the identification of novel XNP mutations in patients with the selected phenotype[8]. We hypothesized that mutations in other regions of the gene could lead to other, possibly less severe, mental retardation phenotypes. We therefore initiated a mutation screening of the entire *XNP* gene by Single Stranded Conformation Polymorphism (SSCP) analysis in patients with nonspecific X-linked mental retardation (MRX). Here, we describe a missense mutation in exon 18 in a family initially diagnosed with mild nonspecific mental retardation. Although on retrospective examination some mild clinical characteristics of ATR-X syndrome could be identified, our results indicate that *XNP* mutation analysis should not be restricted to patients with severe mental retardation and characteristic facial features.

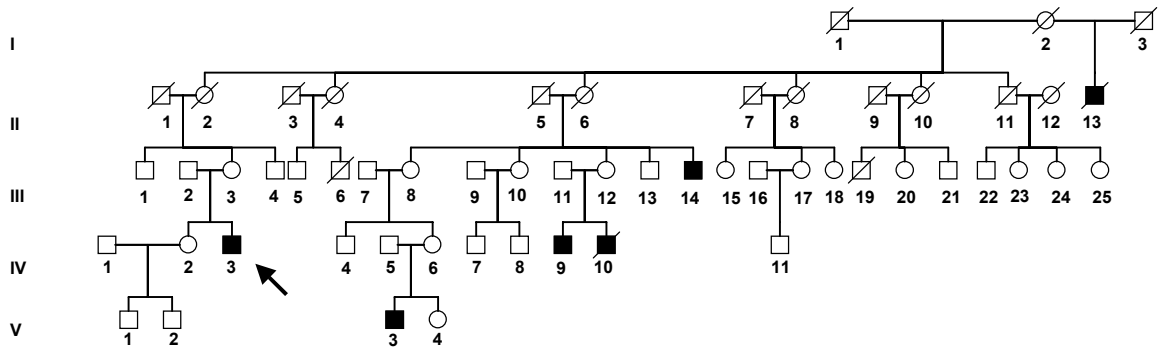

Figure 1. *Five generation family with mild mental retardation with or without childhood hypotonia.*

PATIENTS AND METHODS

Family F96-26

A large family (Figure 1) of Dutch origin with an apparently nonspecific X-linked mental retardation was ascertained when IV-2 was referred for genetic counseling. The pedigree included 7 mentally retarded males in three generations. III-4 had Down Syndrome and was excluded from further investigation. Patients II-13 and III-14 are deceased. In order to determine the mental status of the other 4 patients, the Wechsler Intelligence Scales were used[9,10]. Scores of all tests were transferred into age equivalents according to the classification of the American Association of Mental Deficiency (AAMD)[11]. In the propositus, IV-3, diagnostic tests including cytogenetic analysis, FMR1 mutation analysis, EEG, cerebral CT-scan and ophthalmologic evaluation were all normal. Informed consent was obtained in all instances.

Mutation detection

DNA was extracted from peripheral blood lymphocytes according to the procedure of Miller *et al*.[12]. SSCP analysis was performed on PCR-amplicons of exons 2-4, 6-8, 9 (5' end), 10-22, and 24-27, according to the method of Orita *et al*.[13], with slight modifications. PCR experiments were performed with γ-32^{P}-end-labeled primers as described by Van den Hurk *et al*.[14], in a PTC-100 Programmable Thermal Controler (MJ Research, Inc.). Primer sequences and cycling conditions are available on request. PCR products were denatured and electrophoresed on a 5% nondenaturing polyacrylamide gel[14]. PCR products with an altered electrophoretic mobility were sequenced.

Hematological analysis

Peripheral red blood cells (RBCs) were screened for hemoglobin (Hb) H inclusion bodies. Fresh EDTA-stabilized blood was mixed on a 1:1 ratio with 1% brilliant cresyl blue in 0.9% NaCl. Cells were smeared after incubations at RT for 1 hr and at 37°C for 15 min, 1 hr and 2 hrs. The presence of HbH inclusions in erythrocytes was detected by light microscopy.

RESULTS

Mutation detection

We identified a missense mutation in a patient from family F96-26 (IV-3; Figure 1): 5069C>T in exon 18 giving rise to an amino acid substitution (T1621M) (numbering according to GenBank accession number U75653). The T1621M mutation was not found in 300 control chromosomes, and segregates with the disease in the family over 5 generations with a LOD score of 3.90 at $\theta=0$. This family was clinically diagnosed with nonspecific X-linked mental retardation. No abnormal facial characteristics were detected on first examination. After the discovery of a mutation in the *XNP* gene, the four living mentally retarded males were re-examined.

Clinical data family F96-26

No information is available about patient II-13. Pregnancy and delivery were uneventful in IV-3 and V-3. IV-9 and IV-10 were born prematurely, whereas no information is available for patient III-14. Nonprogressive mental retardation was noted in childhood with delayed speech development as a major feature. The degree of mental retardation varied from moderate to borderline. All showed behavioral problems with chaotic behavior and aggressive outbursts. Results of clinical measurements and psychometric studies are summarized in Table 1. The propositus, IV-3, was examined at the age of 38 years. Except for a double left earlobe and full lower lip, facial hypotonia was visible on the pictures taken in childhood (Figure 2B). III-14 (76 years old) suffers from prostate cancer. Examination showed a full lower lip and relatively large ears (Figure 2A). At the time of examination IV-9 was 45 years old. During childhood he was on anticonvulsive therapy, and since 1992 he is known with micturation problems. Cystoscopy showed mild urethral stenosis. Up-slanting palpebral fissures and a full lower lip were noted (Figure 2C). Ophthalmologic evaluation showed strabismus and hypermetropia in IV-9 and his brother IV-10. IV-10 died because of esophageal cancer at age 34 before he could be examined. Pictures show double earlobe of the left ear, slightly prominent ears, a full lower lip, and childhood facial hypotonia (Figure 2D). Reportedly, he was moderately retarded, showed chaotic behavior, normal OFC and a kyphosis. In patient V-3 (29 years old) unilateral hearing loss was detected in childhood. This patient was not available for clinical re-examination, but pictures show a full lower lip and childhood facial hypotonia (Figure 2E).

After the mutation was found, all four living affected males were tested for Hemoglobin H (HbH) inclusions characteristic for ATR-X and *XNP* gene mutations. In three of the 4 affected males (III-14, IV-9, V-3; Figures 2A, 2C, and 2E) HbH inclusion bodies were found. X-inactivation studies showed extremely skewed X-inactivation in all carrier females (>95% inactivation of the abnormal X-chromosome; data not shown).

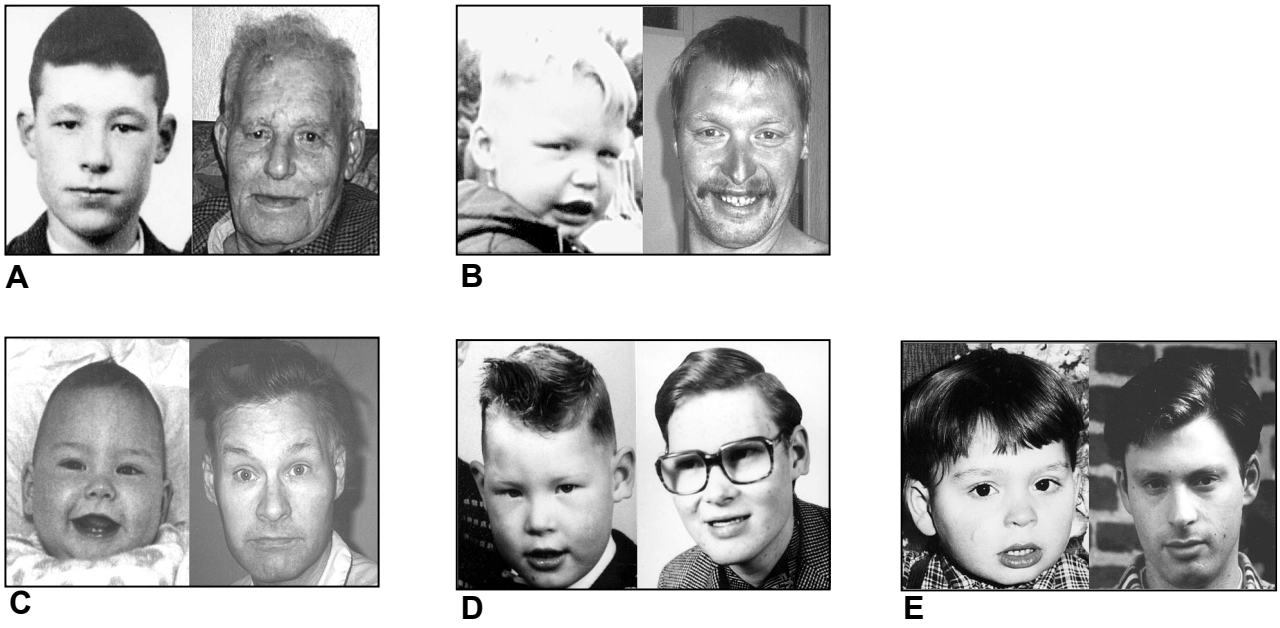

***Figure 2**. Faces of affected males. **A:** Patient III-14 in childhood and at age 76 years; **B:** Patient IV-3 in childhood and at age 38 years; **C:** Patient IV-9 at age 3 months and 45 years; **D:** Patient IV-10 in childhood and at age 34 approximately; **E:** Patient V-3 at age 2 and 27 years. (Photographs reproduced with informed consent).*

***Table 1.** Summary of Clinical measurements (centiles) and psychometric studies*

Patient No.	Age (years)	Height (cm)	Weight (kg)	OFC[a] (cm)	Ear length (cm)	OCD[b] (cm)	ICD[c] (cm)	THL[d] (cm)	Mental impairment[e] (verbal/performal/total)	Adaptive functioning
IV-3	38	178 (10-50)	75 (75-90)	54.3 (3)	6 (50)	9 (50-75)	3.4 (75-97)	19.5 (97)	Mild/mild/mild	mild
III-14*	76	173 (3-10)	60 (25-50)	55 (3-10)	7.2 (97)	9 (50-75)	3.2 (50-75)	19.5 (97)	Mild/moderate/moderate	Moderate-severe
IV-9	45	187 (50-75)	71 (50)	56 (10-25)	6.9 (75-97)	8.3 (10-25)	2.7 (3-25)	19.2 (75-97)	Mild/borderline/mild	Borderline-mild
V-3	29	ND	ND	ND	ND	ND	ND	ND	Mild/borderline/mild-borderline	borderline

ND, not determined
[a]OFC, occipitofrontal circumference
[b]OCD, outer canthal distance
[c]ICD, inner canthal distance
[d]THL, total hand length
[e]Mental impairment: borderline, IQ range 70-84; mild, IQ range 55-69; moderate, IQ range 40-54; severe, IQ range<39
* Due to his current age (76 years) and physical condition, the patient was not capable of performing the tests for adaptive functioning. The intelligence values shown in this table date from age 52 years.

DISCUSSION

We identified a T1621M missense mutation in the *XNP* gene in a patient from a family with generally mild mental retardation and no obvious facial dysmorphisms. Co-segregation of the mutation and the MRX phenotype was observed, with a maximum LOD score of 3.90 at θ=0. Subsequent analysis of red blood cells revealed the presence of HbH inclusion bodies, indicative for disruption of *XNP* gene function. Furthermore, X-inactivation studies revealed extreme skewing of X-inactivation in carrier females, a phenomenon that is typically observed in females carrying an *XNP* mutation.

After the discovery of the mutation, the mentally retarded persons were re-examined to confirm the nonspecific phenotype. Only minor facial abnormalities were found. Although a full lower lip was present in the five affected males described, there is little

resemblance with the facial appearance of ATR-X patients. The facial characteristics are very mild and were not mentioned at the first examination. In three of the patients facial hypotonia was seen on pictures taken in childhood as is usual also for ATR-X, but this was not seen in adulthood. In contrast to other patients with an *XNP* mutation, the degree of mental retardation in this family is borderline to moderate.

The T1621M mutation is located just in front of helicase domain Ia, at a residue conserved with the orthologous mouse gene, but not with that from C. elegans (GenBank accession numbers NM_009530 and AF134186, respectively). Amino acid substitutions in ATR-X patients have been described 7 and 12 residues upstream of the T1621M mutation[1]. In order to get more insight in the function of the XNP protein and to identify possible genotype-phenotype correlations, it is important to search for mutations in additional patients who have mental retardation with and without typical facial characteristics. It was described by Lossi *et al*.[4] that the bias in X inactivation, together with the severe MR, are the only consistent findings in all patients, and that a first selection can be based on these characteristics. The findings in this study, however, strongly suggest that screening of the *XNP* gene must be considered not just in males with severe X-linked mental retardation, but of all degrees of severity.

REFERENCES

1. Gibbons RJ, Picketts DJ, Villard L, and Higgs DR (1995) Mutations in a putative global transcriptional regulator cause X-linked mental retardation with α-thalassemia (ATR-X syndrome). *Cell* **80**: 837-845.
2. Villard L, Gecz J, Mattéi JF, Fontés M, Saugier-Veber P, Munnich A, and Lyonnet S (1996) XNP mutation in a large family with Juberg-Marsidi syndrome. *Nature Genet* **12**: 359-360.
3. Abidi F, Schwartz CE, Carpenter NJ, Villard L, Fontés M, and Curtis M (1999) Carpenter-Waziri syndrome results from a mutation in XNP. *Am J Med Genet* **85**: 249-251.
4. Lossi AM, Millán JM, Villard L, Orellana C, Cardoso C, Prieto F, Fontés M, and Martínez F (1999) Mutation of the XNP/ATR-X gene in a family with severe mental retardation, spastic paraplegia and skewed pattern of X inactivation: demonstration that the mutation is involved in the inactivation bias. *Am J Hum Genet* **65** : 558-562.
5. Stevenson RE, Abidi F, Schwartz CE, Lubs HA, and Holmes LB (2000) Holmes-gang syndrome is allelic with XLMR-hypotonic face syndrome. *Am J Med Genet* **94**: 383-385.
6. Villard L, Fontès M, Adès LC, and Gecz J (2000) Identification of a mutation in the *XNP/ATR-X* gene in a family reported as Smith-Fineman-Myers syndrome. *Am J Med Genet* **91**: 83-85.
7. Guerrini R, Shanahan JL, Carrozzo R, Bonanni P, Higgs DR, and Gibbons RJ (2000) A nonsense mutation of the *ATRX* gene causing mild mental retardation and epilepsy. *Ann Neurol* **47**: 117-121.
8. Villard L, Bonino MC, Abidi F, Ragusa A, Belougne J, Lossi AM, Seaver L, Bonnefont JP, Romano C, Fichera M, Lacombe D, Hanauer A, Philip N, Schwartz C, and Fontes M (1999) Evaluation of a mutation screening strategy for sporadic cases of ATR-X syndrome. *J Med Genet* **36**: 183-186.
9. Van der Steene G, van Haasen PP, de Bruyn E.E.J., Coetsier P, Pijl YJ, Poortinga YH, Spelberg HC, Spoelders R, and Stinissen R (1986) Intelligence scale for children-revised. Lisse: Swets & Zeitlinger.
10. Stinissen J, Willems PJ, Coetsier P, and Hulsman WLL (1970) Wechsler Adult Intelligence Scale, Dutch version. Lisse: Swets & Zeitlinger.
11. Grossman HJ (1977) Manual on terminology and classification in mental retardation. Washington DC: AAMD.
12. Miller SA, Dykes DD, and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**: 1215-1215.
13. Orita M, Suzuki Y, Sekiya T, and Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5**: 874-879.
14. van den Hurk JAJM, van de Pol TJR, Molloy CM, Brunsmann F, Rüther K, Zrenner E, Pinckers AJLG, Pawlowitzki IH, Bleeker-Wagemakers EM, Wieringa B, Ropers H-H, and Cremers FPM (1992) Detection and characterization of point mutations in the choroideremia candidate gene by PCR-SSCP analysis and direct DNA sequencing. *Am J Hum Genet* **50**: 1195-1202.

Summary
Samenvatting
Eenvoudiger gezegd

SUMMARY

With a population incidence of 2-3%, mental handicaps constitute a serious medical and social problem in our society. The causes of mental retardation (MR) are highly variable and involve both genetic and environmental factors. In spite of extensive medical investigations, the etiology of the mental handicap remains unexplained in most patients. It has been estimated that at least 13% of all MR is caused by X-chromosomal gene defects (X-linked mental retardation; XLMR). This means that approximately 400 males with XLMR are born each year in the Netherlands. XLMR can be categorized in syndromic XLMR (MRXS) and nonsyndromic or nonspecific XLMR (MRX). In the latter only the intelligence of the patients is affected. Therefore, identification of the genes involved in MRX will give more insight in the normal development of the brain and particularly of memory formation.

In order to identify genes for MRX, accurate clinical examination and genetic linkage analysis with highly polymorphic markers in MRX families needs to be performed first. To date, in 76 MRX families the genetic defect has been linked to the X-chromosome, two of which are described in this thesis. MRX46 links to Xq25-q26 with a maximum lod score of 5.12 at θ=0 (***appendix 1***), and MRX65 links to the pericentromeric region with a maximum lod score of 3.64 at θ=0 (***appendix 2***).

The MRX linkage intervals are typically too large to directly identify the underlying genes. Moreover, refinement to gene localization is impossible because the individual MRX families cannot be lumped together on the basis of a "common" phenotype. Therefore, other strategies are necessary to identify MRX genes. In general, three methods have been used: positional cloning, (positional) candidate gene analysis, and screening of known MRXS genes.

Positional cloning, using positional information from chromosomal aberrations, has long been the method of choice to identify novel MRX genes. This method has proven its success in the identification of five MRX genes: *FMR2*, *OPHN1*, *TM4SF2*, *IL1RAPL1*, and *ARHGEF6*. *ARHGEF6* is disrupted by a translocation in a mentally retarded boy (***appendix 4***), and a missense mutation was identified in MRX46 (***appendix 1***). However, in some cases no X-chromosomal breakpoint-spanning gene could be identified. In other cases a gene involved in the chromosomal aberration was identified, but no gene-specific mutation in another family could be found to prove its involvement in MRX. An example of this is the *RSK4* gene, that is commonly deleted in mentally retarded patients with a deletion in Xq21 (***appendix 3***). However, no mutations were found in approximately 200 MRX families tested, which means that there is no definite proof of the involvement of *RSK4* in MRX.

Positional candidate gene analysis allowed the identification of two MRX genes: *GDI1* and *PAK3*. Candidate gene analysis may well prove to be effective for the future identification of more MRX genes. For example, it is becoming clear that genes that cause syndromic XLMR with a wide phenotypic spectrum are candidate genes for MRX as well.

Mutations in the genes for Coffin-Lowry syndrome (*RSK2*) and Rett syndrome (*MECP2*) have now also been found in MRX patients. The *XNP* gene, that is involved in ATR-X syndrome and several other severe mental retardation syndromes, is also an excellent candidate gene for MRX. Indeed a mutation has been identified in a family diagnosed with MRX, in which only retrospectively some mild phenotypic characteristics of ATR-X syndrome were found (***appendix 5***).

While the genes involved in MRX encode a variety of proteins, their putative working mechanisms show considerable overlap. Learning and memory are generally affected in MRX. This process involves four stages in the brain: 1) outgrowth of neurons; 2) axonal guidance; 3) formation of synaptic connections, and 4) synapse plasticity leading to long term potentiation. The MRX gene products are part of this mechanism. The outgrowth of neurons starts with the formation of filopodia and lamellipodia, which is mediated by the Rho GTPases Rho, Rac, and Cdc42, which in turn are regulated by the products of the MRX genes *OPHN1*, *ARHGEF6*, and *PAK3*. For the outgrowth of the neurons, vesicle transport mediated by the product of the *GDI1* gene is also essential. The outgrowth of neurons always takes place in a specific direction. This process, called axon guidance, involves repulsive cues (leading to axon collapse) and attractive cues (leading to axon extension). The genes in the mitogen-activated protein kinase (MAPK) pathway downstream of the Rho GTPases, regulated by TM4SF2 and probably FMR2, play a role in axon guidance. After the outgrowth of the correct axons, connections with dendrites of other neurons are formed, which are called synapses. These synapses have to be stabilized and strengthened (synaptic plasticity), which involves neurotransmitter release in the synapses. The product of the *IL1RAPL1* gene is likely to be involved in this release. Finally, a typical human memory is formed, mediated by proteins acting in the nucleus of the neuron, downstream of the MAPKs. RSK2 acts as transcription factor and activates proteins like CREB, which is necessary for long-term memory formation. Besides the above mentioned pathway, epigenetic gene regulation is involved in the formation of memory. MECP2, XNP, and possibly RSK2, play a role in this phenomenon.

The unraveling of MRX genes and the pathways in which they are working paves the way for the identification of novel MRX genes. This will lead to a better understanding of human brain function, and to possibilities for genetic counseling and pre- and postnatal diagnosis.

SAMENVATTING

Met een incidentie van 2-3%, vormen geestelijke handicaps een belangrijk medisch en sociaal probleem in onze maatschappij. De oorzaken van mentale retardatie (MR) omvatten zowel genetische factoren als omgevingsinvloeden. Ondanks uitgebreid medisch onderzoek, blijft de MR in de meeste patiënten onverklaard. Naar schatting wordt tenminste 13% van alle MR veroorzaakt door een gendefect op het X-chromosoom (X-gebonden mentale retardatie; XLMR). Dit betekent dat er in Nederland per jaar ongeveer 400 mannen met XLMR worden geboren. XLMR kan onderverdeeld worden in twee groepen: syndromale XLMR (MRXS) en niet-syndromale of niet-specifieke XLMR (MRX). Omdat een verlaagde intelligentie het enige fenotypische kenmerk is van MRX, zal de identificatie van de betrokken genen meer inzicht geven in de normale hersenontwikkeling, met name in de vorming van het geheugen.

Voor de uiteindelijke identificatie van MRX genen is, naast grondig klinisch onderzoek, koppelingsonderzoek met hoog polymorfe markers in MRX families een eerste vereiste. Tot nu toe zijn er 76 MRX families beschreven, waarin het genetische defect gekoppeld is aan het X-chromosoom. Twee van deze families zijn in dit proefschrift onderzocht. MRX46 is gekoppeld op Xq25-q26 met een maximum lod score van 5,12 bij θ=0 (***appendix 1***), en MRX65 is gekoppeld op de pericentromere regio met een maximum lod score van 3,64 bij θ=0 (***appendix 2***).

De koppelingsgebieden bij MRX zijn in het algemeen te groot om direct met goed gevolg de oorzakelijke genen te identificeren. Bovendien maakt het niet-specifieke karakter van de aandoening het onmogelijk om koppelingsdata te combineren tot een meer nauwkeurige genlocalisatie. Voor de identificatie van de oorzakelijke MRX genen is het gebruik van additionele strategieën dus noodzakelijk. Over het algemeen worden er drie methoden gebruikt: positionele klonering, (positionele) kandidaatgenanalyse, en screening van bekende MRXS genen.

Positionele klonering, waarbij o.a. gebruik gemaakt wordt de positionele informatie van chromosomale afwijkingen, was lang de beste methode om nieuwe MRX genen te identificeren. De methode is succesvol gebleken bij de identificatie van vijf MRX genen: *FMR2*, *OPHN1*, *TM4SF2*, *IL1RAPL1*, en *ARHGEF6*. *ARHGEF6* wordt onderbroken door een translocatie in een mentaal geretardeerde jongen (***appendix 4***), en een missense mutatie werd gevonden in MRX46 (***appendix 1***). Echter, bij sommige translocaties bleek er geen gen te zijn dat onderbroken werd door het X-chromosomale breekpunt. In andere gevallen waar wel een gen betrokken was bij de chromosomale afwijking, kon geen gen-specifieke mutatie in een andere familie gevonden worden die de betrokkenheid bij MRX kon bevestigen. Een voorbeeld hiervan is het *RSK4* gen, dat afwezig is in mentaal geretardeerde patiënten met een deletie in Xq21 (***appendix 3***). Er werden geen mutaties gevonden in ongeveer 200 geteste MRX families, wat betekent dat er geen definitief bewijs is van de betrokkenheid van *RSK4* bij MRX.

Positionele kandidaatgenanalyse heeft geleid tot de identificatie van twee MRX genen: *GDI1* en *PAK3*. Kandidaatgenanalyse zal waarschijnlijk in de toekomst het meest effectief zijn voor de identificatie van MRX genen. Het wordt bijvoorbeeld steeds duidelijker dat genen die betrokken zijn bij syndromale vormen van XLMR met een breed fenotypisch spectrum, goede kandidaatgenen voor MRX zijn. In de genen voor Coffin-Lowry syndroom (*RSK2*) en Rett syndroom (*MECP2*) zijn nu mutaties gevonden bij MRX patiënten. Het *XNP* gen, dat betrokken is bij ATR-X syndroom en verschillende andere syndromen met ernstige MR, is ook een goed kandidaatgen voor MRX. Inderdaad werd een mutatie gevonden in een familie waarbij in eerste instantie de diagnose MRX was gesteld, maar waarbij retrospectief een paar milde fenotypische kenmerken van ATR-X syndroom gevonden werden (***appendix 5***).

Hoewel de MRX genen coderen voor een verscheidenheid aan eiwitten, blijkt het proces van "leren en geheugen" algemeen aangedaan te zijn in MRX. Dit proces in de hersenen kan in vier stadia worden onderverdeeld: 1) uitgroei van neuronen; 2) axon guidance; 3) vorming van synaptische connecties, en 4) synaptische plasticiteit die uiteindelijk leidt tot "long term potentiation". De produkten van de MRX genen maken deel uit van dit proces. De uitgroei van neuronen begint met de vorming van filopodia en lamellipodia, welke gereguleerd worden door de Rho GTPases Rho, Rac, en Cdc42, die op hun beurt gereguleerd worden door de produkten van de MRX genen *OPHN1*, *ARHGEF6*, en *PAK3*. Voor de uitgroei van neuronen is het transport van "vesicles" via het produkt van het *GDI1* gen eveneens essentieel. De uitgroei van neuronen vindt altijd in een bepaalde richting plaats. Dit proces wordt "axon guidance" genoemd en betreft afstotende signalen (leidend tot terugtrekking van het axon) en aantrekkende signalen (leidend tot verlenging van het axon). De genen in de "mitogen-activated protein kinase (MAPK) pathway" downstream van de Rho GTPases worden gereguleerd door TM4SF2 en waarschijnlijk FMR2, en spelen een rol bij "axon guidance". Na de uitgroei van de juiste axonen, worden connecties met de dendrieten van andere neuronen gevormd: synapsen. Deze synapsen moeten gestabiliseerd en versterkt worden (synaptische plasticiteit), wat gebeurt via het vrijkomen van neurotransmitters in de synapsen. Het produkt van het *IL1RAPL1* gen is waarschijnlijk bij dit vrijkomen betrokken. Uiteindelijk wordt een typische menselijk geheugen gevormd, dat gereguleerd wordt door eiwitten die werkzaam zijn in de celkern van het neuron, downstream van de MAPKs. RSK2 werkt als transcriptie factor en activeert eiwitten zoals CREB, dat noodzakelijk is voor de vorming van het lange-termijn geheugen. Behalve de hierboven genoemde route, speelt epigenetische genregulatie een rol bij de vorming van geheugen. MECP2, XNP, en mogelijk RSK2 spelen een rol bij dit fenomeen.

De ontrafeling van MRX genen en de pathways waarin zij werken maken de weg vrij voor de identificatie van nieuwe MRX genen. Dit leidt tot een beter begrip van de werking van het menselijke brein en tot mogelijkheden voor genetische counseling en pre- en postnatale diagnostiek.

EENVOUDIGER GEZEGD

Mentale retardatie, ofwel geestelijke handicap, komt bij 2 tot 3 % van de bevolking voor, en vormt daarom een belangrijk maatschappelijk probleem. Er zijn verschillende oorzaken voor mentale retardatie (afgekort MR), en er zijn zowel erfelijke als niet erfelijke vormen. In sommige gevallen is er een duidelijke oorzaak voor de geestelijke handicap, bijvoorbeeld als er zuurstofgebrek in de hersenen is opgetreden bij de geboorte of als de patiënt het syndroom van Down heeft. Bij de meeste patiënten wordt echter nooit een aanwijsbare reden voor de mentale retardatie gevonden, ondanks uitgebreid medisch onderzoek. Er zijn ongeveer 30% meer mannen dan vrouwen met een geestelijke handicap. Waarschijnlijk wordt dit veroorzaakt door erfelijke vormen van MR, waarbij er een afwijking op het X-chromosoom aanwezig is. Omdat vrouwen twee X-chromosomen hebben en mannen maar één, kan een foutje op dit chromosoom bij mannen tot een ziekte leiden, terwijl vrouwen alleen draagster zijn omdat ze nog een gezond X-chromosoom hebben. Waarschijnlijk wordt meer dan 13% van alle MR veroorzaakt door een afwijking op het X-chromosoom. Deze aandoening wordt geslachtsgebonden mentale retardatie genoemd, afgekort XLMR. Per jaar worden er in Nederland ongeveer 400 mannen met XLMR geboren. XLMR kan onderverdeeld worden in syndromale XLMR (MRXS) en niet-syndromale of niet-specifieke XLMR (MRX). Als er sprake is van een syndroom, hebben de patiënten behalve een geestelijke handicap nog andere afwijkingen waaraan de patiënten herkend kunnen worden (zoals bij Down syndroom). Patiënten met MRX (dus niet-syndromaal) hebben behalve mentale retardatie geen herkenbare afwijkingen. Omdat deze patiënten alleen een verminderde intelligentie hebben, zal opheldering van het foutje op hun X-chromosoom dus inzicht geven in de normale ontwikkeling van de hersenen, en met name in de vorming van het geheugen.

In eerste instantie zal bewezen moeten worden dat de afwijking bij de patiënt daadwerkelijk op het X-chromosoom ligt. De belangrijkste methode hiervoor is koppelingsonderzoek. In eerste instantie wordt een familie, waarin vermoedelijk MRX voorkomt, grondig klinisch onderzocht, en blijven alleen die families over waarin er meerdere aangedane mannen zijn die op basis van IQ-testen een geestelijke handicap blijken te hebben, en waarin er geen andere kenmerken zijn die wijzen op een syndroom. Bovendien moet het duidelijk zijn dat de ziekte in de familie doorgegeven wordt via draagsters, d.w.z. moeders die wel het foutieve X-chromosoom hebben maar zelf niet zijn aangedaan omdat ze nog een gezond X-chromosoom hebben. Daarna wordt in het laboratorium het DNA van zoveel mogelijk familieleden onderzocht. Er wordt een soort kaart gemaakt van de X-chromosomen van alle personen. Wanneer een bepaald gedeelte van deze kaart overeenkomt in alle patiënten, terwijl alle gezonde familieleden dat gedeelte niet hebben, wordt het waarschijnlijk dat het foutje bij de patiënten in dit gedeelte van het X-chromosoom ligt. Men zegt dan dat de afwijking "gekoppeld" is aan dit gedeelte van het X-chromosoom. In dit proefschrift is in twee Nederlandse families zo'n

koppelingsgebied op het X-chromosoom gevonden (***appendices 1 en 2***). Wereldwijd zijn er nu 76 MRX families beschreven met zo'n koppelingsgebied op het X-chromosoom.

In families waarin een koppelingsgebied is gevonden kan geprobeerd worden in dit gebied een afwijking in het DNA te vinden dat de mentale retardatie veroorzaakt. Deze afwijking moet zich dan bevinden in een gen: een klein gedeelte van het DNA dat de erfelijke code bevat. De koppelingsgebieden die gevonden worden in MRX families zijn echter over het algemeen erg groot. Bovendien blijkt het dat iedere familie zijn eigen koppelingsgebied heeft, dat niet altijd overlapt met het gebied van een andere familie. Dit betekent dat de geestelijke handicap in de ene familie een hele andere oorzaak heeft dan in de andere familie, en dat er dus meerdere genen op het X-chromosoom betrokken zijn bij mentale retardatie. Men verwacht dat er meer dan 50 genen op het X-chromosoom zijn die een rol spelen bij ontwikkeling van het geheugen bij mensen. Gelukkig zijn er andere manieren dan koppelingsonderzoek, om in een kleiner gebied, te zoeken naar genen voor MRX: positionele klonering, (positionele) kandidaatgenanalyse, en screening van bekende MRXS genen.

Positionele klonering is een methode waarbij in een heel klein gebied op een chromosoom naar een gen gezocht wordt. Zo'n nauwkeurige localisatie is alleen mogelijk als er een structurele fout op het chromosoom is, bijvoorbeeld als een stukje van het X-chromosoom uitgewisseld is met een ander chromosoom (translocatie), als er een stukje van het X-chromosoom afwezig is (deletie), of als er een stukje van het X-chromosoom is omgedraaid (inversie). In ***appendix 3*** van dit proefschrift is gezocht naar een gen in een stuk DNA dat bij een aantal mentaal geretardeerde mannen afwezig was. Bij gezonde personen ligt op dit stuk DNA een gen, het *RSK4* gen, dat waarschijnlijk noodzakelijk is voor de ontwikkeling van een normale intelligentie. We konden echter niet definitief bewijzen dat dit gen echt betrokken is bij mentale retardatie, omdat er nog een ander gen in dit stuk DNA kan liggen. Als we een foutje in dit gen alleen hadden gevonden in een andere familie (dus zonder deletie), konden we definitief bewijzen dat dit gen een MRX gen was. We hebben meer dan 200 families getest, maar geen afwijkingen gevonden. Voor vijf andere genen die met de positionele kloneringsmethode zijn gevonden kon dit definitieve bewijs wel gevonden worden. Eén van deze genen is beschreven in ***appendix 4***. Dit gen, *ARHGEF6*, wordt onderbroken door een translocatie in een mentaal geretardeerde jongen. In de Nederlandse familie van ***appendix 1*** werd een hele andere fout in hetzelfde gen gevonden, waarmee het bewijs dat dit gen betrokken is bij geestelijke handicaps rond was.

Twee MRX genen zijn geïdentificeerd door middel van positionele kandidaatgen analyse. Bij deze methode wordt gezocht naar genen die op basis van hun functie een rol zouden kunnen spelen bij mentale retardatie. Daarna wordt gekeken in welk gebied op het X-chromosoom zo'n kandidaatgen ligt en worden de families gezocht waarbij koppeling in dit gebied is gevonden. Nu door het Humane Genoomproject het hele DNA van de mens in kaart gebracht wordt en bijna alle genen op het X-chromosoom bekend zijn, zal deze kandidaatgen analyse steeds belangrijker worden. Bovendien is gebleken dat

er bij MRX ook foutjes gevonden kunnen worden in genen die een syndroom kunnen veroorzaken. In de genen voor Coffin-Lowry syndroom (*RSK2*) en Rett syndroom (*MECP2*) zijn nu mutaties gevonden bij MRX. Het blijkt dat ernstige fouten in deze genen, waarbij het gen helemaal niet meer functioneert, tot een syndroom kunnen leiden, terwijl minder ernstige fouten, waarbij het gen slechts minder functioneert, tot MRX kunnen leiden. Het *XNP* gen, dat betrokken is bij ATR-X syndroom, en verschillende andere syndromen met ernstige MR, is ook een goed kandidaatgen voor MRX. Inderdaad werd een mutatie gevonden in een familie waarbij in eerste instantie de diagnose MRX was gesteld (***appendix 5***). Achteraf gezien blijkt dat de patiënten in deze familie toch wat kenmerken van het ATR-X syndroom bezitten, en dat we dus eigenlijk niet van niet-specifieke mentale retardatie mogen spreken.

Het is niet alleen belangrijk om de genen te vinden die betrokken zijn bij MRX, maar ook om te weten waarom een foutje in zo'n gen leidt tot geestelijke handicap. Wanneer men kijkt naar de functie van de genen die inmiddels gevonden zijn bij mentale retardatie, valt op dat de meeste van deze genen een rol spelen bij de ontwikkeling van de zenuwcellen in onze hersenen. Nu er negen genen voor MRX zijn geïdentificeerd en er een idee is over het mechanisme waarop geheugen bij de mens wordt gevormd, wordt het waarschijnlijk makkelijker om nog meer genen voor MRX te vinden. Dit leidt tot een beter begrip van de werking van het humane brein. Bovendien worden de mogelijkheden voor erfelijkheidsadvies en pre- en postnatale diagnostiek vergroot als er meer genen voor MRX ontdekt worden.

DANKWOORD

Nu mijn proefschrift klaar is, en ik nog eens terugkijk naar alle resultaten die ik de afgelopen jaren heb geboekt, blijkt duidelijk dat ik veel mensen dank ben verschuldigd. Eigenlijk hebben alle collega's van de afdeling Antropogenetica in Nijmegen door de gezellige ongedwongen werksfeer bijgedragen aan het slagen van dit proefschrift. Uiteraard zijn er mensen die meer dan een algemeen bedankje verdienen:

Hans, nu ben je voor het eerst co-promotor! Bedankt voor alles wat je me hebt geleerd en voor het vertrouwen in een goede afloop (dat ik zelf lang niet altijd had). Door het geroffel van je toetsenbord (af en toe onderbroken door wat gezang), wist ik dat je altijd op de achtergrond aanwezig was om mijn talloze vragen te beantwoorden. Hilger, bedankt dat je me de mogelijkheid hebt gegeven om bij Antropogenetica te promoveren. Ik ben blij dat je we via het Europese consortium nog steeds over het XLMR onderzoek kunnen praten. Han, het leek soms of je mijn gedachten kon lezen! Je positieve benadering en je heldere kijk op mijn onderzoek zorgen ervoor dat er niet meer ellenlang gediscussieerd hoeft te worden over wie wat wanneer en vooral hoe op gaat schrijven! Frans, bedankt voor je bijdrage aan met name het Xq21 werk. Jammer dat het bewijs voor een MRX gen in "jouw" gebied nog niet helemaal rond is.

Het mentale retardatie onderzoek op onze afdeling zou niets zijn zonder de aanwezigheid van Ben. Samen met Francis, met de orthopedagogen (eerst met Tanja en nu met Anja), met DNA-diagnostiek (Erik en Willy), en met Tjitske en Bert als meest recente versterking zullen ook in de toekomst nog veel leuke resultaten verkregen worden. Bellinda, jouw enthousiasme in het lab werkt aanstekelijk! Misschien wel een beetje té..... (maar de champagne was wel erg lekker!). Astrid, gelukkig was jij er om de boel draaiende te houden toen ik in Frankrijk was, en toen ik aan mijn proefschrift aan het werk was. Zonder jou zouden er helemaal geen resultaten meer verkregen worden op dit moment! Johan, bedankt voor je pogingen om mij de fijne kneepjes van het moleculaire werk bij te brengen, en vooral voor de gezelligheid en de humor die je op het lab bracht. Gerard, ik hoop dat het niet aan mij lag dat je zo snel vertrokken was toen je eenmaal aan MR moest werken! Ik ben blij dat je inmiddels weer terug bent op het lab.

Ook wil ik de studenten bedanken die direct of indirect aan mij werden toevertrouwd: Freek, Mieke en Esther, bedankt voor jullie bijdrage en voor de gezelligheid! Verder heeft de rest van de vakgroep Moleculaire Genetica natuurlijk bijgedragen aan een leuke werksfeer: Ellen, Jacopo, Pascal en Betsie, het is rustig maar wel erg gezellig aan onze kant van de muur! Dorien, Anneke, José, Stef, Saskia, Marc, Martijn, Wendy, Hannie, Peter, Yvette, Alessandra, Janneke, Sioe Lie, Carolien, Marga en Sylvia, allemaal bedankt voor de gezellige koffie- en theepauzes en voor de kletspraatjes tussen het pipetteren door!

I want to thank the collegues of the Mandel group at the IGBMC in Strasbourg for the nice time I had in your lab. Especially André and the RSK2 group, Maria, Sylvie, Karine and Solange: it was really nice working with you! Maria, you are a great friend,

without you my stay in France would have been less fun! Furthermore, I am indebted to our collegues of the European XLMR consortium. The exchange of knowledge and patient material was, and still is, very important for the mental retardation research in Nijmegen.

Buiten het werk zijn mijn familie en vrienden onmisbaar gebleken. Ik wil mijn ouders en Reinier en Marceline bedanken voor jullie onvoorwaardelijke steun en interesse in mijn onderzoek. Monique en Rianne, mijn wekelijkse etentje met jullie was (en is) altijd een welkome afleiding. Marinke, je hebt jarenlang als mijn thuisfront gefungeerd: ik denk echt dat ik het zonder jou niet had gered!

Ronald, dankzij jou heb ik mijn promotie zonder al te veel stress af kunnen ronden. Gelukkig heb je meer verstand van computers dan ik! Ik ben blij dat je met me meedacht als het nodig was, maar vooral dat je ervoor hebt gezorgd dat ik niet meer overal over hoef na te denken (al kan ik dat soms toch niet laten). Nu ook mijn boekje klaar is, kunnen we echt eens gaan doen waar we zin in hebben!

CURRICULUM VITAE

Helger IJntema werd op 10 mei 1971 geboren in Eindhoven. Na het behalen van het eindexamen Atheneum aan het Anton van Duinkerkencollege in Veldhoven, begon zij in 1989 aan de studie Biologie op de Katholieke Universiteit Nijmegen. Gedurende deze studie deed zij een hoofdvak Experimentele Dierkunde bij Dr. Wim Scheenen, waarbij de receptor voor Neuropeptide Y in de pars intermedia van *Xenopus laevis* werd gekarakteriseerd. Vervolgens werd een bijvakstage Neurobiologie gedaan aan de Universiteit van Torino, Italië (onder leiding van Prof. Dr. Aldo Fasolo), waar immunohistochemisch onderzoek werd gedaan aan de bulbus olfactorius van de muis. Tenslotte werd een tweede hoofdvakstage gevolgd op de afdeling Moleculaire Hematologie, begeleid door Dr. Jules Meijerink, waarbij de identificatie van *Bcl2*-gelijkende genen centraal stond. Na het behalen van het doctoraalexamen Medische Biologie in 1995 heeft zij een jaar als wetenschappelijk onderzoekster in het Klinisch Chemisch Laboratorium van het Medisch Spectrum Twente in Enschede onderzoek gedaan naar het groeigedrag van niet-kleincellige longtumoren. Sinds 1 maart 1996 is zij als wetenschappelijk onderzoekster werkzaam op de afdeling Antropogenetica van het Universitair Medisch Centrum St. Radboud in Nijmegen, waar het in dit proefschrift beschreven onderzoek is uitgevoerd. Tijdens haar promotie-onderzoek verbleef zij vijf maanden in het lab van Dr. André Hanauer op het Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in Illkirch (Strasbourg), Frankrijk.

PUBLICATIONS

Yntema HG, Oudakker AR, Kleefstra T, Hamel BCJ, van Bokhoven H, Chelly J, Kalscheuer VM, Fryns J-P, Raynaud M, Moizard M-P, and Moraine C. In frame deletion in *MECP2* causes mild nonspecific mental retardation. *Am J Med Genet*, in press.

Couvert P, Bienvenu T, Aquaviva C, Poirier K, Moraine C, Gendrot C, Verloes A, Andres C, Le Fevre AC, Souville I, Steffann J, des Portes V, Ropers HH, Yntema HG, Fryns JP, Briault S, Chelly J, and Cherif B (2001) *MECP2* is highly mutated in X-linked mental retardation. *Hum Mol Genet* 10: 941-946.

Kutsche K, Yntema H, Brandt A, Jantke I, Nothwang HG, Orth U, Boavida MG, David D, Chelly J, Fryns J-P, Moraine C, Ropers H-H, Hamel BCJ, van Bokhoven H, and Gal A (2000) Mutations in *ARHGEF6*, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation. *Nat Genet* 26: 247-250.

Yntema HG, van den Helm B, Kissing J, van Duijnhoven G, Poppelaars F, Chelly J, Moraine C, Fryns J-P, Hamel BCJ, Heilbronner H, Pander H-J, Brunner HG, Ropers H-H, Cremers FPM, and van Bokhoven H (1999) A novel ribosomal S6-kinase (*RSK4; RPS6KA6*) is commonly deleted in patients with complex X-linked mental retardation. *Genomics* 62: 332-343.

Yntema HG, van den Helm B, Knoers NVAM, Smits APT, van Roosmalen T, Smeets DFCM, Mariman ECM, van der Burgt I, van Bokhoven H, Ropers H-H, Kremer H, and Hamel BCJ (1999) X-linked mental retardation: evidence for a recent mutation in a five-generation family (MRX65) linked to the pericentromeric region. *Am J Med Genet* 85: 305-308.

Yntema HG, Hamel BCJ, Smits APT, van Roosmalen T, van den Helm B, Kremer H, Ropers H-H, Smeets DFCM, and van Bokhoven H (1998) Localisation of a gene for non-specific X linked mental retardation (MRX46) to Xq25-q26. *J Med Genet* 35: 801-805.

Scheenen WJJM, Yntema HG, Willems PHGM, Roubos EW, Lieste JR, and Jenks BG (1995) Neuropeptide Y inhibits Ca^{2+} oscillations, cyclic AMP, and secretion in melanotrope cells of *Xenopus laevis* via a Y_1 receptor. *Peptides* 16: 889-895.